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The Morphological Determination of the Retothelial System and its Pathogenetic Importance

By

O. Fresen

From the Pathological Institute, Medical Academy, Düsseldorf, Germany; Director : Prof. Dr. H. Meessen

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I.

The concept of the reticulo-endothelial system (Aschoff) follows from the histological evaluation and analysis of organic functions. This system is firstly regarded as a physiological entity (Pasteur Vallery-Radot)[69]. Its functions are, however, associated with morphological changes which very well show the relationship between function and morphology. Its multifarious manifestations-linked with storage and phagocytosis, interrelated with the intermediate metabolism, elicited by reactive and inflammatory processes, and associated with polymorphic neoplasias-make it desirable to examine the orthological structure of its changes morphologically, to differentiate them histologically and classify them nosologically. Only with such knowledge it is possible to draw reliable conclusions on the importance of this system, the pathological significance of which was emphasized only recently (Policard)[73].

Since this system is not an organ in the conventional meaning of the word, only a synoptic evaluation of individual experiments and pathologico-anatomical findings, proved by histological examination and electron microscopy, can lead to an understanding of its morphology.

II.

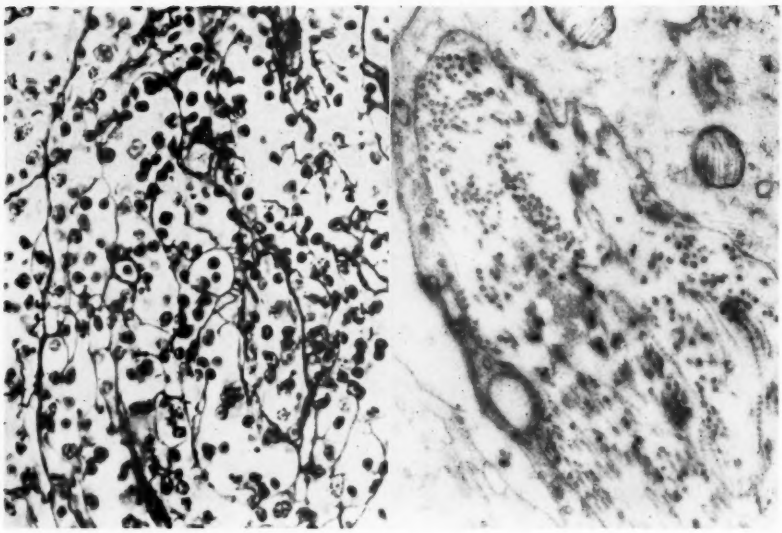
The postulation of this cell system resulted from animal experiments conducted to determine cellular behaviour in inflammation (Metschnikoff, Kiyono)[50] and from observations on absorptive (Landau[54] and McNee) and excretory (Suzuki)[93] processes. At first it was possible only to apply the results to human pathology as an analogy requiring further verification because, in spite of the great similarity of functions in man and animals, the morphological integrity of such a cell system, whose postulation can be based only on a specific outlook, is not necessarily the same (Henschen)[40]. Performing a causalanalytic experiment

is a very difficult problem in medicine, particularly in human pathology, as its interpretation is too easily affected by an inclination to draw relative conclusions. However, if, for example, an infusion of polyvinyl pyrrolidone evokes in man and experimental animals the same picture of kollidon storage, so far as position and amount are concerned (Fresen and Weese)[27], then it provides a basis for such an analytical experiment. It also allows a proper evaluation of the results of storage experiments with cerebrosides (Kimmerstiel and Laas)[49] and sphingomyelin (Beumer and Gruber)[13] with regard to thesauropathies in man, and the avoidance of misleading analogous conclusions in understanding and differentiating this cell system.

A. *The reticular cell component* of this functionally evaluated system is determined by the lympho-reticular tissue, spleen and bone marrow.

The structure of the reticular cell tissue is fairly simple; it shows ramifying, interconnected cells which in quiescence have a clear and unstructured plasma with an apparatus of Golgi near the nucleus. The relatively large, and often lacunar nucleus contains little chromatin, but usually possesses a nuclear body and a distinct nuclear membrane. These cells are little characteristic interstitial cells which on light-microscope examination give the impression of a syncytial configuration. In this cellular compound fibrils occur which are closely related with the exoplasm (Studnicka)[52] and form the spatially oriented network of the lattice fibres. They differ from the collagen fibres of ordinary connective tissue by their fineness, their property of tinging on plasma staining, and their characteristic-although non-specific-ability to take up silver. The latter is not an artefact, as can be seen by direct phase-difference microscopy of reticular cell tissue. This fibrillary reticulum shows a uniform structure which determines the structure of lymph nodes, tonsils, spleen and bone marrow (Fig. 1a). As the cell stroma of the lympho-reticular tissue also outside the lymph nodes it harbours the lymphocytes and the myeloic blood cells in the reticulum of the bone marrow; in the spleen it forms the spongy blood-conducting pulp.

Electron-microscope studies confirmed that the reticular cell is a large, multi-branched and only slightly characteristic cell which, according to its functional needs, exhibits a comparatively variable minute structure (Fresen and Wellensiek)[28]. The increase in mitochondria and ergastoplasm lamelles and vacuoles begins the transformation to active macrophages. Distinct, layered lamelles of ergastoplasm and formation of rather varied coacervations indicate the differentiation to plasma cells; the homogenous, translucent amorphous contents of the lacunae in the endoplasmatic reticulum can then form in to Russel's bodies, or condense to ball- or rod-shaped formations, or organise in polymorphous



a) b)

Fig. 1. *Reticular tissue.*

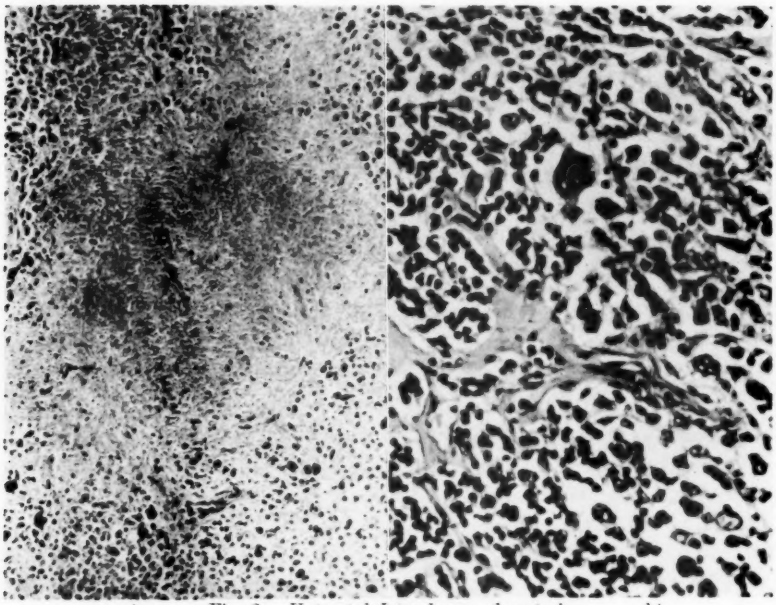
a) Framework of the argyrophil reitculum in lymphnode (silverimpreg. 500×)
b) fascile of long and transverse cutted fibrils uearly enclosed by a reticulum cell. (rat, lymph node, 16400×2).

crystals (Wellensiek)[100]. Investigations of homogenisates (Lindner)[58] led to the assumption of trajectory differentiation of the symplasmatic configuration to fibrils, which appear only in the ectoplasmic region and which, when shadowed, show aperiodic external silver uptake. In contrast to the light-microscope impression of a syncytial structure, in the submicroscopic range the reticular cell tissue shows continuous cleft-like cell borders which, because of the marked spatial interrelationship of the cells, form a bizarre system of joints. The continuous intercellular clefts widen to intercellular spaces of very different shapes, up to 0.8 μ wide. In these clefts and lacunae run longitudinal and cross-striated fibrils. With a medium thickness of 200 to 400 Å they are not always sharply differentiated from the interfibrillary homogenous, or scarcely granulated, ground substance. Their transverse striation shows a periodicity of about 610 Å; splitting is not recognizable in them. Several fibrils run together, either straight or slightly undulated, and separate around groups of equally ball-shaped formations. Cross sections show pale circular discs with a slightly densified margin, about 60 Å wide. These fibrils together form an oriented, obviously non-ramifying bundle, reaching the dimension of a lattice fibre visible, when silverblackened, by light-microscopy. This bundle has been confirmed to be the fine structure of the reticular

fibres also by its differences from collagen fibre (Schwarz)[85] seen on electron microscopy. As these intercellular spaces, filled with fibres, are occasionally almost completely surrounded by the cytoplasm of one of the various reticular cells, they may give a false impression of being intracellularly situated (Fig. 1b). This appearance can be understood at first only as the plastic adjustment to local conditions. However, it is often possible to demonstrate a course of the fibrils in which at least a close relationship to plasma extensions from individual reticular cells cannot be overlooked. This observation of the relationship of fibrils to plasma, which is not imitated by spatial superimposition, may contribute to the discussion on the actual place of formation of the filaments. This has been assumed to be at the surface of the cell (Porter)[74]. Observations that the precursors of collagen fibrils are expelled from the fibroblasts in the form of unarranged packs (Weiss[99]; Gieseking)[32] may be of importance in this connection. They tend to support the hypothesis that the site of fibrillogenesis in the ectoplasma, which separates out as structurally still not differentiated protofibrils (Wassermann)[98]. This is not improbable since heterogenous filaments are formed in the plasma of various cells. It may be that the two modes alternate in the formation of reticular fibrils, in depending on the colloid-chemical conditions in the intercellular groundsubstance or the functional state of the reticular cells.

The reticular fibre is not, therefore, a transitory phase in the development of the typical collagen fibre in a fibrocytic milieu. The lattice-fibre structure remains a non-varying characteristic of reticular cell tissue in normal conditions; its hyalination occurs only under disturbed conditions. Also differences in function (Bargmann)[8] and composition (Missmahl)[63] allow to distinguish the argyrophil fibre from the collagen fibre. The argyrophil fibre in fibre-bundle form remains a special structural feature of the reticular cell tissue, which characterises also its morphologically observable manifestations. Such a lattice-fibre structure occurs also in inflammatory tissue proliferation of reticular cells to granuloma- for example, in the tubercle (Fresen)[21]- and also determines the fine structure of retothelial sarcoma (Roulet)[81].

The *follicles* of spleen and lymphatic tissue are a characteristic, but in their structure only little differentiated, part of the reticular cell component. They consist of cells which are rich in plasma and in varying degree of approximation and usually form a distinct net without formation of argyrophil fibres. Their varying behaviour with regard to occurrence and disappearance, cell density and number of mitoses caused to postulate various phases of formation, according to functional requirements (v. Albertini[3]; Ono)[66]. The active, loosened follicle, which shows plenty of mitoses, consists of large macrophagocytic elements



a) Fig. 2. *Untreated Lymphogranulomatosis.* b)

a) recent necrosis with surrounding granulation tissue.
(S.-Nr. 265/56, 15y. male; H.-Eos. 110×).

b) typical polymorphic substratum with STERNBERG's -cell and incipient hyalinisation.
(E.-Nr. 1834/53, 29♀. male; H.-Eos. 310×).

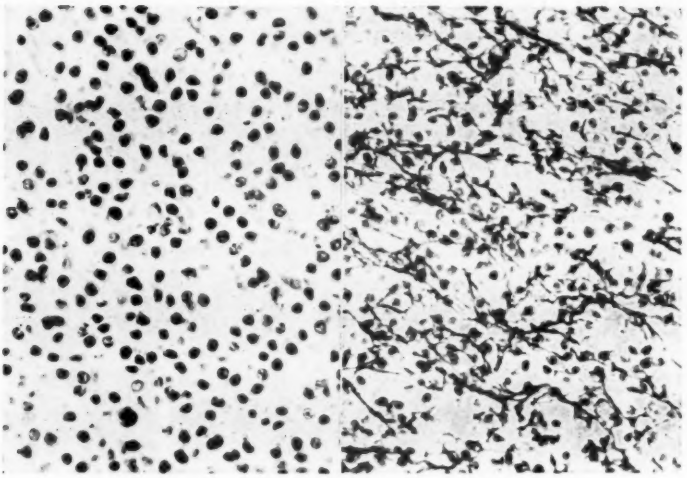
(Marchand).[59] They form in animal experiments with polyvinyl pyrrolidone (Fresen and Weese)[27] or cerebrosides (Kimmerstiel and Laas)[49] and in Gaucher's disease (Pick)[70] distinct nets of storage cells around the capillaries, which, when replaced by lattice fibres, may remain connected with the follicle cells. The same conditions and changes are found in the follicles of the lymph nodes. The nodule in Boeck's disease with the dense net of argyrophil fibres, which is unusual for the locality (Fresen)[22], develops from the follicle cells which have undergone epitheloid transformation; these also take part in the histological processes in infectious mononucleosis with hyperplasia and increase in fibres, and mobilisation of the cells (Gall and Stout)[30].

B. The *endothelial component* of the system originally included the sinus cells of lymph nodes and spleen and the endothelium of the liver capillaries, blood capillaries of the bone marrow, suprarenals and pituitary. It was later extended to the endothelium of the thymus and all the blood vessels.

In the lymph nodes the mural cells of the lymph paths are also connected with each other and with the surrounding reticular cells by lattice fibres and, therefore, may be considered to be endothelially positioned reticular cells according to flow conditions. Because of these findings, which also apply to sections of a similar structure in the blood vessels, the reticulum of the spleen and bone marrow (v. Herrath)[41], these parts of the lymph and blood vessels of the reticular cell tissue are termed *sinusoids*. The loose structure of their walls differs markedly from the endothelium of ordinary blood and lymph vessels bordered by a basal membrane. The stellate cells of the liver form the cellular component of a lattice fibre tube and, therefore, show the structural characteristics of sinusoids. Their different appearance prevents them from being classified as ordinary endocytes (Zimmermann)[103]. Their transformation into storage cells and the formation of reticular-fibrillary nodules in abdominal typhoid and in hepatitis prove their reticular cell character, which is also seen in animal experiments in the conversion of stellate cells, tagged with dyestuffs, into epitheloid cells of the tubercle. Even in reticulosis the proliferating stellate cells imitate the structural principle of reticular cell tissue. Their function and appearance therefore, on the whole, correspond to those of the reticular cells.

Under the *electron microscope* it is possible to mistake the true nature of the cells bordering the sinusoids, where the reticular cells arrange to coating cells without bordering basal membrane. Submicroscopically continuous clefts show between both the extensions of the regularly flat forms and the bulging activated cells. They begin at the surface of the sinusoid as a pore, occasionally slightly funnel-shaped, and are continuous with similar clefts between adjacent reticular cells (Fresen and Wellensiek)[28]. As it has been possible to see intercellular clefts between the border cells of the rat's spleen (Weiss)[99], between the overlapping Kupffer cells in the liver of mouse (Parks)[68] and rat (Rüttner and Vogel)[83], the sinusoids lined with retothelial elements may be assumed to share a common structural principle. The same structure is found in the sinusoids of spleen and liver in man (Stoeckenius;[91] Cossel[17]). In large surfaces this common structural principle may explain the ease and rapidity with which storage and phagocytosis occur, and, because of the absence of an obturating basal membrane, would also be of special significance in explaining permeability in the region of the lining cell compound (sinusoids).

According to the results of dyestuff-storage experiments in animals (Kiyono)[50] corresponding properties are ascribed to the *endothelium of the blood capillaries* of the bone marrow, the suprarenals, hypophysis and also the thymus, and also applied to humans. However, the histological findings in Gaucher's disease and in polyvinyl pyrrolidone storage and their experimental equivalents do not indicate



a) b)

Fig. 3. *Myelom*. (S.-Nr. 699/50; 54y. female)
a) tissue of plasmacells (H.-Eos. 310×).
b) with fibrillary structure (silverimpreg. 310×).

an involvement of the capillary endothelia in these organs. Besides the corresponding conversion of reticular cells in the bone marrow and thymus, the storage cells develop in distinctly pericapillary situations; the endothelial cells, regularly provided with a basal membrane, remain unchanged. This fact is of importance in the determination of the extent of the endothelial component of the system, since the findings resulting from changes bound to a system are not biased by analogy conclusions and possible misinterpretations.

In view of the discrepancy in the attempt to include the whole vascular endothelium in the endotheloid component of the system (Törö)[95], storage experiments were carried out on healthy, untreated rabbits, rats, and mice, which were sensitized against heterogenous serum and also others treated with diphtheria toxin and histamine in order to increase capillary permeability. The results of these experiments were compared with those obtained in polyvinyl pyrrolidone storage in animals (Fresen)[21]. In dyestuff-storage experiments it has to be borne in mind that the degree of storage is dependent on doses and time; as well as on the dispersing properties of the test material and the varying conditions of capillary permeability. These experiments, in which all the various factors were taken into consideration, showed that even with maximum doses neither the general vascular endothelium in whatever organ or tissue nor the capillary endothelia of the bone marrow, suprarenals, hypophysis and thymus are involved in the

storage functions of the cells of this system. Iron-sugar in colloidal solution, which is often used in these storage experiments, causes deposits at the bordering surfaces which, like the irregular deposits and micro-embolies from large-molecular ink, cannot be explained as endothelial storage. It was only the misinterpretation of the histological findings in this actually mechanical process which gave rise to the- apparently functionally founded- inclusion of the whole capillary endothelium into the storage-cell system.

The endothelial cells of a typical capillary wall neither possess, nor assume, the function or form of a reticulogenic border cell. The principal differences between ordinary endothelial cell and reticulogenic border cell in both function and structure cannot, therefore, be overlooked. The vascular endothelium of regular structure thus does not belong to the endothelioid component of the system.

C. This exclusion is valid also for the capillary endothelium of the lung. The storing elements-Gaucher's cells-occurring in the septa and alveoles, and the cells of desquamative catarrh, or the cellulation of the alveolar tubercle are not of endothelial origin but develop pericapillary in and from the septa. These mesodermal elements clearly differ also cytologically from the alveolar epithelium. They are derived, like the storage cells and phagocytes in connective tissue, from the vascular *histiocytes* (Kiyono). This explaines that the storage capacity of the endothelium, supposed to occur under the local action of histamine, is a result of the greater activation and mobilisation-following increased capillary permeability- of the adventitially and pericapillary present, or developing, storing and phagocytic macrophages: the permeability-increasing effect of histamine, by increasing histiocytic storage, leads to a more rapid elimination of the administered material from the blood (Symkovics, Kiss and Fekete). These wandering cells have a function similar to that of the primary fixed reticular cells and the mobile reticulogenic border cells of the sinusoids (Omori)[64]. With their fibrillary texture, typical of that seen in the joining together of reticular cells, they clearly show their common origin, with the reticular cell component of the system (Akazaki) in the histological formation to secondary nodule (Hurt)[46] and granuloma (Fresen)[22] and in the autonomous proliferation occurring in a primary retothelial sarcoma (Roulet)[81] outside the system in its original meaning. This system is found, in the form of histiocytes, also in the leptomeninges and the central nervous system. The pericapillary histiocytes store injected dyes directly and subarachnoidally to a much higher degree than the microglia cells from which they morphologically differ, but which occasionally were also included in the system (Onishi)[65]. This explains the essentially histiocytic-reticular determined productive phase corresponding to localised tuberculous and lymphogranulomatous

processes (Wepler)[101]; granulomatous encephalitis, which is considered to be a virus infection (Wilke)[102], the perivascular infiltrations in the meninges and the brain substance in infectious mononucleosis (Allen and Kellner)[4], and the primary retothelial sarcoma in the brain (Gerhartz)[31].

These histiocytes also form the *intima granulomas* of various origin which develop, independently of the endothelium, in the subendothelial zone of the intima. Here, the histiocytic storage cells were found both in animal experiments (Hueper)[44] and kollidon storage in man (Hüsselmann[45], Poche)[71–72]. Activation of this subendothelial germinal layer, which is also present in the small arteries, can be shown by the effect of pharmaceutical substances, which increase permeability (Waters and Duff).

D. This cell system can therefore be summarised to consist of the fibrillary compound of reticular cells, including the endothelioid positioned reticulogenic border cells of the sinusoids in spleen, lymph nodes, bone marrow and liver, and of the histiocytes with corresponding functions and form. The functionally and histomorphologically founded exclusion of the endothelium of the blood and lymph paths led to its being termed the *retothelial system* (Fresen)[21]. Its fibrillary basic structure is a constant histological feature which is maintained or formed in either reactive, inflammatory or neoplastic processes. This proves the histological identity of the reticular cells, the border cells and the histiocytes, and the integrity of the R.S. as a non-differentiated remainder of the foetal mesoderm of multifarious prospective significance.

III.

On this histomorphological basis the *pathogenic importance of the R.S.* becomes evident in metabolic, imflammatory and neoplastic changes in localised areas or in the whole system. They have been discussed in detail elsewhere (Fresen)[21].

A. Histologically demonstrable changes already develop under abnormal strain and in derangements of organic *function* which vary according to localisation. It is sufficient just to mention them, since the pathology has been discussed only at recent symposia (Halpern *et al.*[34]; Heller[39]).

The importance of the R.S. in the degradation of erythrocytes and thus in the formation of bile pigment and in iron metabolism (Vanotti[96]) has been confirmed. It is particularly involved in the various forms of haemolytic disease associated with hypersplenism (Doan[20]). The *spleen* as the most important organ of the system exerts with the formation of hypoxylienin and a hemopoietic substance hormone-like distant actions, which are also seen in the splenomegalic inhibition of blood formation in the bone marrow (Heilmeyer[38]). The degrada-

tion of *globulins* in the lympho-reticular tissue was proved in animal experiments (Schallock[84]). The intake and metabolism of antigenic proteins is connected with the formation of various defence substances (Jansco[48]) and antibodies, including those against heterogenous vaccination tumours (Hoepke[43]) which are digested by histiocytes (Szabadi[94]). In storage experiments, in which high demands are made on the R.S., anaphylactic shock fails to occur (Klinge); at the same time the development of immunity recedes. Macroglobulinaemia is accompanied by hyperplasia of the reticular cells of the bone marrow and lymph nodes, and is suggestive of protein formation in the cells of the system. In cystin disease, associated with dwarfism and renal osteodystrophy, cystin crystals are stored in all parts of the R.S. and by the histiocytes (Roulet)[81]. A disturbance in *fat-metabolism* of apparently cellular origin (Letterer)[57] is the endogenous cause of generalized lipid-storage disease, as in Gaucher's disease and Niemann-Pick disease. The circumscribed histiocytic storage of cholesterol esters leads to the formation of xanthelasma, and cholesterolaemia to generalized xanthosis (Bremer and Lübbers[14]). Also the role of the system in the metabolism of *carbohydrates* and their storage and conversion was confirmed in animal experiments (Lindner[58]). Melanin, fatty substances, and hemosiderin are found in the histiocytes of the corium and the external lymph nodes-rarely also in the bordering cells of spleen and liver - in various skin diseases of completely different aetiology and pathogenesis. This lipomelanosis is a distinctly reactive, reversible process (Meessen[61]). Further, for proper *vitamin metabolism* (Brown, Philips and Kawan)[15] and growth (Manzini and Menini[60]) this system is of importance. The decrease of storage capacity (Frimmer[29]), phagocytosis (Heller[39]), shedding of the cells of the system (Bena-Cerraf *et al.*[10]) and formation of antibodies (Squire) under the action of ACTH or cortisone show without doubt that the system is also subject to *hormonal influence*, but the influence of the nervous system was not yet been clarified (Shiochi *et al.*).

B. In *inflammatory reactions* only the mobile elements of the R.S. are involved at first - the histiocytes, which form macrophagocytic nodules in cases of living or inanimated foreign body. Desquamative catarrh of the lung is due to alveolar phagocytes mobilised from the septae. Sinus catarrh in lymph nodes originates from an increased rate of shedding of the coating cells, which in infectious mononucleosis can lead to monocytic blood changes. In human poliomyelitis nodular reactions of reticular cells with gaint cells were found not only in the lymph nodes but also in spleen and other lympho-reticular tissue (Sommers *et al.*[89]). The histomorphological substrate in tuberculosis (Akazaki *et al.*[2]), Boeck's disease (Fresen b), tularaemia and typhus (Randerath)[76], listerosis (Reiss[79]), viral lymphadenitis (Hedinger[37]), etc. is essentially characterised by *granulomas*. The epithelioid elements often formed from the local reticulum or

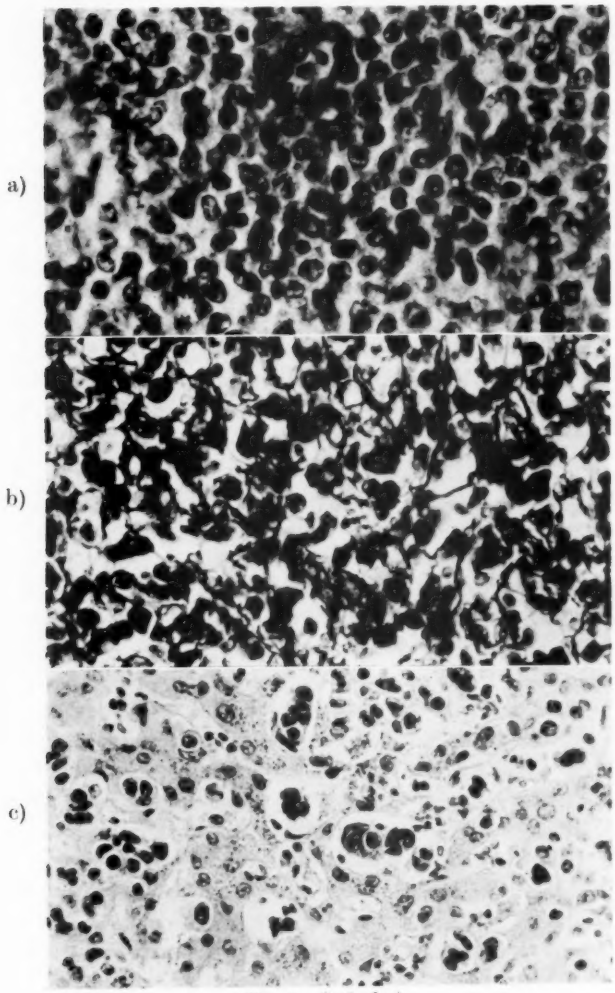


Fig. 4. *Reticulosis.*

a) proliferation of the reticulum cells (H.–Eos. 200×)
b) formation of an irregular net work of lattice fibres. (silverimpreg. 250×) (S.–Nr. 113/48, 19y, female)
c) multiplication and desquamation of monocytoid KUPEFER's cells. (H.–Eos. 200×) (S.–Nr. 764/42, 45y. male).

the vascular germinal layers are in varying degrees connected with ordinary granulation tissue and occasionally also with eosinophil granulocytes.

Lymphogranulomatosis is considered to be the prototype of a disease determined by granuloma. The progressing dissemination, especially in the lymph node groups, gives a macroscopic impression of tumours, a fact which has been the reason for ascribing to Hodgkin's disease a neoplastic character. The giant-celled granuloma is the decisive histological finding and only it allows to coordinate the anatomical findings to the entity of the disease. The phasic development of the granuloma, through sclerosis of its lattice fibre structure and degenerative atrophy of its cell element to fibrotic nodule, should be considered, in relation to the reversibility of progressive and regressive processes, as inflammatory in nature. This arouses interest in determining the early changes. Sinus catarrh and the fine structure of so-called para-granuloma could be well understood as the initial changes, in spite of their non-specificity. However, since they remain confined to lymph nodes, they cannot be thought to be of fundamental importance in the determination of generally valid early changes. Of decisive importance in the histogenesis of the lymphogranulomatous processes and the nosological classification of the disease is the spontaneous softening (Sternberg[90]); it represents direct necrobiosis of the affected place, and this precedes the granuloma. It is much more likely to be the primary damage- although of unknown aetiology - of the local tissue, since it is not bound to the lympho-reticular tissue. The invariably progressive sequence of granuloma and fibrosis is supplemented by the initial alteration of the tissue for the regular phasic course of inflammation (Fresen[23]). This is confirmed by the quantitatively extreme histological conditions with extensive tissue necroses in acute lymphogranulomatosis. Owing to the often rough-morphologically tumour-like appearance and the histological monomorphy in the later sclerotic stage, the histology of lymphogranulomatosis led to the assumption of a neoplastic process (Jackson and Parker)[17]. Absence of corresponding histological findings in the necrobioses of lymphogranulomatous foci, however, disprove a pre-existent neoplasia with local inflammatory reaction. Occasionally observed genuine tumours in lymphogranulomatosis are sporadic events without statistic significance and non-pathognomonic of the nature of the disease. By their regular and largely uniform histological findings they differ from the course and varied form of the lymphogranulomatous changes. A tumourous character of lymphogranulomatosis cannot be concluded even from the rough-morphologically impressive destructions (Uehlinger[97]). The inflammatory nature of lymphogranulomatosis does not, however, at the same time exclude the possibility of its malignant degeneration to sarcoma, but it is not known whether and how often such tumourous conversion into a blastomatous form may occur in lym-

phogranulomatosis.

In a similar way the inflammatory nature of *eosinophil granulomas of bone* is shown by a reticulo-histiocytic phase, and of *lipoid granulomatosis*, which mainly occurs in children, by the secondary xanthelasmatic conversion of the granulomatous foci (Letterer)[57].

Histological examinations of diseases marked by granuloma showed, that the histioid structure of the cell reaction, independent of the localisation, gives rise to a typical-reticular fibre network in which also the giant cells are involved as remaining non-differentiated symplasms of reticular-histiocytic origin. The sequence of tissue reactions characteristic of granulomas shows that the reticulo-histiocytic granuloma is inflammatory in nature and corresponds, therefore, to a specially formed granulation tissue. In view of its various causes, the granuloma cannot be generally explained as an allergic reaction which is less specific of a largely isomorphic correlation but due to a particular cause.

C. *Neoplasias* of a reticular cell matrix form a more uniform histomorphological group. They show however some peculiar features in that they may occur ubiquitously and disseminate, in the form of so-called histohomologous metastases, preferably in tissue milieus related to them, develop multicentrically, or as a generalised disease show relations to hemoblastoses.

Amongst them, *retothelial sarcoma* is considered to be the example of an autonomous proliferation of reticulo-histiocytic cells because it imitates the texture of the retothelial matrix. It can occur solitary or multiple (Kojima[53]) and also develop from the undifferentiated vascular zones, and a primary tumour may be found outside the true R.S. in various organs, including the brain. The reticular type of Ewing's sarcoma corresponds to a retothelial sarcoma situated in the bone marrow.

Myelomas, because of their uniform fine structure and with their own lattice fibre formation, should be considered in the main to be reticulogenous plasma cell tumours of various stages of maturity (Fresen[21]). Extraossal metastases are relatively rare ; primary extramedullary tumours were seen in the respiratory and gastric mucosa. From observation of the behaviour of plasmocytomas it is also possible to prove their retothelial origin by demonstrating the antibody formation which is bound to the R.S. and often associated with an increase of plasma cells; this antibody formation is bound to those globulins which occur in paraproteinaemia, e.g. in gamma-plasmocytoma. Here the relationship of autonomous cell proliferation to the metabolic functions of the system is as evident

as in *macroglobulinemia*, which is accompanied by a proliferation of the reticular cells.

The relationship of *Brill-Symmers disease* to the retothelial neoplasias has been proved by the determination of a generally valid histological substrate and its pathogenesis. The relatively rare disease, taking a protracted course and being associated with sometimes considerable increase of lymph nodes and/or the spleen, can disseminate to such an extent as to give the impression of a systemic disease. The even macroscopically visible nodules correspond to follicle-shaped cell proliferations, which may densely spread throughout the organ and suppress the original local tissue. In spite of their resemblance they are not follicles. The cells have relatively little plasma, and in loose position show a reticular structure. Their structure and the varying formation of fibres which take up silver show the reticular cell character of these proliferations. Because of the different explanations of this typical substrate as a reaction product of various causes or a lymphatic tumour capable of differentiation the disease can be recognized only by its pathogenesis. Therefore, the reticuloma may be conceived to be a manifestation of the R.S. in the form of a prospective malignant neoplasia, whose preference for lympho-reticular tissue for its growth and spread appears to be milieu-conditioned (Fresen). With its peculiar course, and presenting only little symptoms the disease ends with tumour cachexia as a result of the multicentric onset of catapasia in the formation of retothelial sarcoma.

Reticulosis is a primary generalised proliferation of cells of the R.S.. It affects all age groups; in children it is often attended by temperatures and skin changes, which is the reason why it is referred to as Abt-Letterer-Siwe disease in this form. Its onset is non-characteristic; the sysmptom resemble largely those seen in leukoses; in some cases there are disturbances of protein metabolism resembling those in plasmocytoma. This disease, which can take an acute or chronic course, affects practically all organs and tissues; spleen, liver, bone marrow and the lympho-reticular tissue in its closer sense are always affected; the involvement of the bone marrow can lead to local destructions or cyst-like translucencies of the bone; its localisation in the skin is associated with various changes including multiple node formation and ulceration; the infiltrations do not even spare the central nervous system. The various anatomical changes are histologically paralleled in proliferation of the local reticular cells and/or histiocytes with separation of the proliferated border cells and formation of distinctly polymorphic giant cells. Involvement of the undifferentiated zones of the vessels with separation of, or penetration through, the endothelium may also lead to intima granuloma. A similar proliferation can be produced in animals with trypan blue (Papacharalampous[67]; Rüttner and Brunner[82]). The rough morphological

pattern often corresponds to that of a nodular leukosis, and when multiple tumour formation occurs it cannot be clearly differentiated from disseminated retothelial sarcoma. The attempt at a nosological classification of reticulosis can only be made with the knowledge of the various morphological manifestations of the reticulo-histiocytic tissue. Changes due to metabolic causes can be differentiated from the homogenous cell picture of reticulosis by their form and the demonstration of the stored substances by staining and chemical methods. The two agree as lipoidoses only in their spread which is partially bound to the system. Reticulosis differs from infective changes by its progressive course, and from granuloma by its constantly uniform histology. These general pathological findings rule out the tuberculous aetiology in reticulosis (Cazal[16]) and any theory of an infectious cause (Sternberg[90]). The irreversible character and uniform histomorphological substrate of this systemic proliferation distinguish reticulosis principally from local and extensive reactions due to metabolic or infectious causes and also from tumour-like manifestations of the R.S.. These characteristics therefore rule out the possibility of regarding reticulosis as a neoplasia of the R.S. (Deelman[19]) and mark it as a autonomous proliferation of the total R.S. (Fresen[21]; Klemperer[51]). It is seen that not all more or less generalised and retothelial determined diseases can be termed reticulosis. The retothelial determined diseases should therefore be stated by their own names when their aetiology is known and they can be distinguished by their clinical symptoms, findings, and course and prognosis. Therefore there is absolutely no reason to include other diseases, uncritically grouped together as so-called lymphoid tumours (Berman[12]), in reticulosis and also to standardise them as reticuloses.

D. If the R.S. is to be considered, according to its form and functions, as the little differentiated remainder of the mesoderm, which in fetal development also is the place of blood cell formation, then its *connections with hemopoiesis* should be mentioned. They are referred to in detail elsewhere (Fresen[25]).

Definite hemopoiesis corresponds, as a regeneration from late fetally determined stem-cells, does not correspond to the primary blood cell formation. While erythro- and granulocytopoiesis remain bound to the reticular cell marrow tissue, lymphocytes, monocytes and plasma cells are formed chiefly extramedullary in lympho-reticular tissue and the vascular adventitia. Also the extramedullary hemopoiesis occurring in various blood disease prefers the sinusoids of spleen and liver as sections of the R.S. It therefore remained to verify histomorphologically the assumption of a myeloic metaplasia with regard to the structure of reticulo-histiocytic tissue formations.

Hyperplastic processes, as an intensification of the physiological model, can

often give a clue to orthological conditions. In congenital hemolytic *anemias*, for example, the reactive hyperplastic erythropoiesis persists in its places of formation and also leads to a displacement of marrow functions into extraossal situations, namely the maturation of precursors prematurely eliminated from the marrow. Also genuine *polycythemia* cannot be considered a neoplastic disease of the bone marrow originating from the reticulum because of the chiefly intravasal spread of the normally autonomous proliferation in the marrow; no histological evidence of erythroblastic differentiation of the extraossal local retothelium is available as it does in genuine leukemic erythroblastosis (Fresen[25]).

Animal experiments showed that also local formation of *granulocytes* in extramedullary places is possible in the sinusoids (Apitz[6]) and the adventitial germinal layer (Herzog[42]). This mode, which is probable also for man (Hamperl[35]), is a possible local function of the R.S.. Extramedullary foci are rarely seen in infectious myeloic-leukemoid reaction (Seige and Janssen[86]). Thus, if they are not always present, then the immature blood picture cannot be due to extramedullary myeloic metaplasia (Rohr[80]), but indicates hyperplastic medullary myelopoiesis. Also adequate animal experiments (Fresen and Lierenfeld[26]) have shown that their retothelial origin is improbable, in spite of their preference for the sinusoids. For normal *myelosis* no histomorphological evidence is available of genetic relations to the local retothelium in either marrow or extramedullary foci. The localisation of leukotic foci can therefore be thought to indicate only a uniform dissemination of the blood cell proliferation, obviously due to the special milieu in the R.S.. Independent and localised accompanying retothelial proliferations mark the picture of *myeloreticulosis* (Benecke[11]).

The fine structure of the compensatory *extraossal foci of blood formation* in osteopetrosis (marble-bone disease) or osteomyelosclerosis does not prove their local development in the sense of the repeatedly postulated myeloic metaplasia; as an intensified regeneration it corresponds pathogenetically to a reactive hyperplasia. Spontaneous foci of blood formation of marrow type are important findings for clarifying local blood cell formation. Owing to their reticular cell stroma, and being bound to adipose tissue, they correspond to an organoid ectopy of the marrow. Since these foci are not a partial occurrence of a general extramedullary reactive or neoplastic blood cell formation, both persistence of fetal hemopoiesis and colonisation of accidentally displaced stem-cells can be excluded in their development; their metaplastic origin, proven by their fine structure, indicates a hemopoietic function of the polyblastic reticular cells originally formed from the vascular germinal layers (Fresen[25]).

The cellulation of granulation tissue suggests a local direct development of lymphocytes, monocytes and plasma cells. Animal studies indicate for *lymphocytes* the development from the reticular cell stroma of the lymphoreticular tissue (Amano[5]; Grundmann[33]). Also in man, lymphocytopoiesis is bound to a reticular cell matrix (Lennert and Remmele[56]). This histomorphological feature is not found in lymphocytic neoplasias; the stroma is not involved in the proliferation of its parenchyma in lymphatic *leukosis*. An accompanying reticular cell proliferation is found as lymphoreticulosis (Apitz[6]), but is rare.

With the increase in ergastoplasm and formation of Russel's bodies in a potently developed endoplasmatic reticulum proceeds the change in form from reticular cell to *plasma cell* (Stoeckenius[91]; Wellensiek[100]). The development from the local portion of the R.S., proved for plasmocytoma, is suggestive of a metaplastic development also of *plasma cell leukosis*, which in 31.4 % of cases is associated with myeloma. Its histological substance was confirmed to be a reticulosis (Klüken and Preu[52]). Waldenström's disease is obviously a special form of such retothelial leukosis with metabolic manifestations (Zollinger).

The *monocyte* is a reticulogenic blood cell of a histocytic nature. Monocytic transformation, especially of the border cells, was proved by electron microscopy also in infectious mononucleosis (Reinauer[78]). The monocytic reaction is the hematological expression of an activation of the R.S. *Monocytic leukosis* is based on the histological substrate of a generalised proliferation of the R.S. in the form of reticulosis, as has been repeatedly confirmed (Poche and Stüttgen[72]). An identical behaviour is seen in reticulosis of the rat which can be transmitted by inoculation (Harris *et al.*[36]).

The R.S. retains post-natally hemopoietic potencies as a remainder of the fetal mesoderm. The demonstration of the retothelial substrate and transitory hematological and histological forms also revealed proliferations of the R.S. in various states of hemopoietic differentiation; leukosis and reticulosis are the pathogenetic principle of retothelial hemoblastosis, which is the pathological image of mesodermal blood cell formation.

IV. SUMMARY

In Kyoto 1924 Aschoff has suggested, that future researchs would prove, whether it has been justified to unite different but nevertheless cooperating cells to a special system. The conclusions of physiological and pathological examinations just as microscopical and submicroscopical investigations have substantiated, that this retothelial system, at first only functionally established, is characterized by a common morphological substratum.

This cell system consists, in a closer sense, of the provinces of the reticular cell tissue which forms with its net of argyrophil fibres the stroma of lymphoreticular tissue and bone marrow, and the structural element of the spleen. Reticular coating cells are the endotheloid elements lining the sinusoids of the bone marrow, spleen, liver and lymph nodes; the essential feature distinguishing them from the general vascular endothelium is the absence of a basal border by a collagen basement membrane. The extended portion of the system is formed by mobile histiocytes in the undifferentiated circumvasal and subendothelial zones, where they can also be formed. Submicroscopically the reticular cell tissue, including the lining cells, show cell borders and continuous intercellular clefts which, owing to the spatial connection of the cells, communicats with the sinusiods. Cross striated fibrils are seen in the amorphous ground-substance of the widened clefts; as oriented bundles they correspond to the histological argyrophil reticular fibre. These fibrils occur not only extracellularly but sometimes are connected with the ectoplasm of the reticular cells, which has to be considered for the mode and place of fibrillogenesis. This system, which was first conceived from the agreement in functions of its elements, is seen to have an adequate morphological structure, which is maintained in both reactive and inflammatory manifestations of the system and is formed in the various neoplasias. These findings, when compared with the fine structure of fetal connective tissue, are an indication of the apparently slight differentiation of this cell system. This remainder of the embryonal multipotent mesoderm can be termed the retothelial system, since the ordinary endothelium of the blood-and lymph-parts can be excluded for its form and functions.

On the examples of granulomatoses and neoplasias it is discussed, that the uniform texture of the reticular histiocytic tissue formations is the essential prerequisite for explaining the pathogenesis and prospective importance also of such retothelial determined diseases, whose origin is not yet known and whose nosological classification is still disputed. Demonstration of the retothelial substrate, including the cells in this stroma, and observation of developmental forms by cytological and histological examination also allowed to determine connections between the origin of regular and morbid hemopoiesis and the retothelial system. A synoptic evaluation of the findings presented confirms the anatomical integrity of the R.S. and its multifarious importance in health and disease.

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Blood Pictures in Stomach Cancer, Especially on Their Relation with Cancer Progress

Second Report : Red Cells and Hemoglobin

By

Iwao Yamaguchi and **Shozo Okuguchi**

From Prof. M. Muto's Surgical Clinic, School of Medicine, Tohoku University, Sendai

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Anemia is, no doubt, one of the manifest signs common to all malignancies. Recently, a statistical analysis of 1628 stomach cancer cases in our clinic has revealed that the anemia is observed in about 70 per cent of stomach cancer cases, that the rate of occurrence as well as the severity of the anemia is somewhat related with the extent of the cancer spreads and the size of the tumor, and that the anemia in stomach cancer has a close relation to the gastric hemorrhage and to the acidity of the gastric content.[1)]

In the work to be reported here, the hematological studies have been performed in stomach cancer cases and in the controls more precisely, giving the results essentially the same as above, as shown in the following.

EXPERIMENTAL AND COMMENTS

METHOD

The objects for the study were 71 cases with stomach cancer (50 males and 21 females; aged 21 to 75 years, 54.5 years on an average). The researches were performed also in 20 healthy adults and in 26 cases with peptic ulcer of the stomach and/or duodenum, for comparison.

Red cell count (RC) and reticulocyte count (Ret.) were carried out by the usual methods, while the hemoglobin concentration (Hb) was estimated as follows : the whole blood was poured into 100 volumes of distilled water containing 0.03 per cent potassium ferricyanide and 0.02 per cent potassium cyanide, and allowed to hemolyze, and hemoglobin cyanide thus appeared in the supernatant was measured by the spectrophotometry at 540 mμ. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) were calculated in the usual manner.

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RESULTS

Normal Values

The results with 20 healthy beings are shown in Table I. Referring to these, the terminology for the present study was determined as follows: "anemic", in case when either RC was less than 4,000,000 per mm^3 of blood or Hb was below 13.0 g/dl; "micro-, normo-, and macrocytic", when MCV was less than 86, 86 or more but less than 104, and 104 μ^3 or more, respectively; "hypo-, normo-, and hyperchromic", when MCH was less than 28, 28 to 36, 36 $\gamma\gamma$ or more, respectively.

TABLE I. Results with Healthy Adults

	Minimum	Maximum	Mean*	Rejection limits (at α=0.05)
RC ($\times 10^3/mm^3$)	3,910	5,960	4,830±200	3,980–5,680
Hb (g/dl)	12.8	17.4	15.4±0.3	13.0–17.7
MCV (μ^3)	83.9	104.9	95.3±1.0	86.5–104.1
MCH ($\gamma\gamma$)	28.5	37.3	31.9±0.5	27.8–36.0
Ret. (‰ of RC)	3	16		

* With 95 per cent confidence ranges.

Results wtih Stomach Cancer Cases

The hematological values in stomach cancer are shown in Table II, in contrast with those of the ulcer cases. RC and Hb in cancer of the stomach were thus proved to be difinitely lower even when compared with those in peptic ulcer, a non-malignant disease of the same organ. Distribution ranges of both MCV and MCH were comparatively large in stomach cancer, though their means were almost equal to those in peptic ulcer, respectively. Ret. were found to be too divergent to be studied further.

TABLE II. Results with Stomach Cancer and with Peptic Ulcer Cases

Cases		Minimum	Maximum	Mean
Peptic ulcer (26 cases)	RC	3,520	5,720	4,430±270
	Hb	11.3	19.0	14.3±0.8
	MCV	83.5	121.1	98.8±3.3
	MCH	27.0	38.9	32.4±1.2
	Ret.	3	27	
Stomach cancer (71 cases)	RC	2,360	5,700	4,050±160
	Hb	6.6	17.1	12.2±0.6
	MCV	68.6	125.6	95.5±7.1
	MCH	20.3	37.8	30.2±0.9
	Ret.	1	26	

TABLE III. Distribution of Various Types of Anemia in Stomach Cancer

	Microcytic	Normocytic	Macrocytic	Total
Hypochromic	8 (17.0%)	10 (21.3%)	1 (2.1%)	19
Normochromic	2 (4.3%)	19 (40.4%)	5 (10.6%)	26
Hyperchromic	0	0	2 (4.3%)	2
Total	10	29	8	47

Anemia was observed in 47 (66.2 %) of the stomach cancer cases and in only nine (34.6 %) of the peptic ulcer cases. It was also noticed that the "anemia" in the above-mentioned sense was due, in stomach cancer, mainly to the decreased Hb, whereas in peptic ulcer almost equally both to the decreased RC and to the decreased Hb.

Table III shows the types of anemia in the mentioned 47 cases with stomach cancer. The types were various as expected from the widely diverged MCV and MCH, though the normocytic-normochromic cases were commonest among them, in accordance with Oppenheim's observation.[2] This probably suggests that the anemia in stomach cancer may be of many different origins.

Relaion with the extent of cancer progress (Table IV)

The rate of occurrence of the anemia was increased with the progress of the

TABLE IV. Relation of Red Cell Pictures in Stomach Cancer with Extent of Cancer Progress

Cancer spreads*		No. of total cases	Mean values				No. of cases with anemia
			RC	Hb	MCV	MCH	
Peritoneal dissemination	P.C. O, I	37	4,180±240	12.8±0.9	96.9±3.5	30.7±1.4	19(51.4%)
	II, III	34	3,900±210	11.6±0.8	94.0±2.3	29.7±1.3	28(82.4%)
Lymph-nodal involvement	L.M. O, I	20	4,290±310	13.2±1.2	97.2±3.6	30.8±2.1	9(45.0%)
	II, III	46	3,980±200	11.9±0.8	94.8±3.2	30.0±1.2	33(71.7%)
Stage of progress	Stage I	18	4,440±300	13.6±1.0	97.4±4.1	30.8±1.8	7(38.9%)
	II	42	3,940±220	11.7±0.7	94.1±3.4	29.7±4.1	31(73.8%)
	III	11	3,710±630	12.0±1.5	97.5±4.6	31.4±2.3	9(81.8%)

* For explanations, see Table II of the First Report.[3]

TABLE V. Types of Anemia for Various Extents of Cancer Progress

Stage of progress	No. of cases	Micro-cytic	Normo-cytic	Macro-cytic	Hypo-chromic	Normo-chromic	Hyper-chromic
Stage I	7	1	4	2	3	4	0
II	31	8	18	5	14	15	2
III	9	1	7	1	2	7	0

lesion. It should be noticed, however, that about a half of the total was anemic even in cases with only localized dissemination or in those with little involvement of the lymph nodes. In Table V, the types of the anemia are shown for various grades of cancer spreads.

Relation with morphology of the main tumor

The results are given in Table VI and VII. The rate of occurrence of the anemia as well as the mean Hb was significantly related with the size of the tumor, but far less with macroscopic or microscopic features of the main tumor.

TABLE VI. Relation of Red Cell Pictures in Stomach Cancer with Morphology of the Tumor*

Morphology+		No. of total cases	Mean values				No. of cases with anemia
			RC	Hb	MCV	MCH	
Size	Small	16	4,460±270	13.9±1.0	98.1±5.6	31.4±6.8	6(37.5%)
	Medium	16	3,830±400	12.0±1.6	98.5±4.5	31.2±1.6	11(68.8%)
	Large	31	4,010±250	11.6±0.9	92.3±3.7	28.9±1.6	23(74.3%)
Gross appearance	Circumscribed	52	4,020±200	12.2±0.8	96.2±2.8	30.4±1.1	34(65.4%)
	Infiltrative	11	4,340±330	12.5±1.4	91.0±6.8	28.9±3.0	6(54.5%)
	Ulcerated	53	4,060±200	12.1±0.7	94.8±2.5	29.8±1.0	36(67.9%)
	Non-ulcerated	10	4,150±450	13.2±2.1	98.2±1.3	31.8±3.7	4(40.0%)
Histological feature	Adenomatosum	26	3,870±270	11.9±1.2	96.8±4.3	30.4±1.8	19(73.1%)
	Solidum	37	4,190±240	12.5±0.8	94.7±3.3	30.0±1.3	22(59.5%)

* In 63 cases subjected to resection of the stomach.
\+ For explanations, see Table III of the First Report.[3]

TABLE VII. Types of Anemia for Various Sizes of the Tumor

Size of the tumor	No. of cases	Microcytic	Normocytic	Macrocytic	Hypochromic	Normochromic	Hyperchromic
Small	6	2	3	1	4	1	1
Medium	11	1	6	4	2	9	0
Large	23	6	15	2	12	10	1

Relation wih the extent of gastric hemorrhage

In Table VIII, "occult" means that the bleeding was so light as could be recognized first by the benzidine test of the stool, and "manifest" shows the evident one resulting in blood vomiting or in the tarry stool. Though anemia was found most commonly in cases with the manifest bleeding, it was observed also in a half of the cases without any clinical evidence of gastric hemorrhage. This may show that the bleeding from the tumor is indeed a predominating but not the single cause of the anemia in stomach cancer.

TABLE VIII. Relation of Red Cell Pictures in Stomach Cancer with Gastric Hemorrhage

Grade of bleeding	No. of total cases	Mean values				No. of cases with anemia
		RC	Hb	MCV	MCH	
No	6	4,360±400	12.7±2.8	88.6±13.9	29.1±5.5	3 (50.0%)
Occult	47	4,130±190	12.7±0.7	96.7±2.8	30.6±1.1	29 (61.7%)
Manifest	18	3,710±360	11.1±1.4	94.8±4.5	29.7±2.1	15 (83.3%)

SUMMARY

Red cell pictures were studied in stomach cancer cases with special reference to their relation with morphological aspects of the tumor, with the results as follows:

1) Anemia was observed in 47 (66.2 %) of 71 stomach cancer cases. The anemia in stomach cancer differed from that in peptic ulcer, not only in the rate of occurrence but also in some other features.

2) The type of the anemia was various in stomach cancer, suggesting different origins of the anemia.

3) Of various aspects of the lesion, its extent of the spreads as well as the tumor size was most closely related with the occurrence of the anemia in stomach cancer..

4) Bleeding from the tumor was found to be a significant but not the only factor resulting in the anemia in stomach cancer.

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Tohoku J. Exper. Med., 1960, 73, 27-30

Blood Pictures in Stomach Cancer, Especially on Their Relation with Cancer Progress

Third Report : Serum Calcium

By

Iwao Yamaguchi, Tsunemasa Suzuki and **Ryoichi Motoki**

From Prof. M. Muto's Surgical Clinic, School of Medicine, Tohoku University, Sendai

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Changes in the serum sodium and potassium content in stomach cancer have been clarified by many authors including those in our clinic.[1)2)] In the present communication will be reported the results of serum calcium analysis.

Needless to say, calcium is contained in the serum in two main forms, one is the ionized or dialyzable fraction responsible for the physiological activities of serum calcium and the other is the combined calcium regarded as a depot of the former. These two fractions were treated separately in this work.

EXPERIMENTAL AND COMMENTS

METHOD

The objects for this study were 68 cases with stomach cancer (46 males and 22 females, aged 26 to 65 years), as well as 20 healthy adults and 23 cases with peptic ulcer of the stomach and/or duodenum for comparison. The quantitative analysis of serum calcium was carried out by Yanagisawa's method,[3)] as follows:

1) *Total calcium.* A 0.1 cc portion of the serum was diluted with 0.4 cc of redistilled water, to which were added 2.0 cc of 0.00025 M/1. 1-hydroxy-4-chloro-2,2-diazobenzene-1,8-dihydroxy-naphthalene-3,6-disulfonic acid disodium salt (Plasmo Corinth B, Sumitomo) and 2.5 cc of 2*N* sodium hydroxide. After three minutes, the mixture, a red transparant solution, was subjected to the photometry at 634 mμ, against the blank solution prepared similarly but using redistilled water instead of the serum. From the transmittance thus obtained, the concentration of total calcium was determined on the standard curve prepared previously.

2) *Combined calcium.* The procedure for measuring the concentration of the combined calcium was essentially the same as above except that a 4.0

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per cent solution of ammonium oxalate was used in place of redistilled water as the diluent of the serum. At that time, Plasmo Corinth B was added exactly three minutes after the serum had been mixed with the oxalate solution.

3) *Ionized calcium.* The concentration of the ionized calcium (hereafter to be referred to as Ca^{++}) was calculated as the difference between those of the total and the combined calcium. The ionization quotient (I.Q.) was also calculated for reference, as Ca^{++}/total Ca.

RESULTS

Normal Values

The mean values in 20 healthy adults were 9.6 ± 0.3 mg/*dl* for total Ca, 4.4 ± 0.2 mg/*dl* for Ca^{++}, and 0.46 ± 0.01 for the I.Q. From the individual values obtained, the normal range of total Ca^{++} and Ca was calculated as an area surrounded by the ellipse having the formula[4]

$$0.98\,(x-9.6)^2-2\times0.37\,(x-9.6)(y-4.4)+2.84\,(y-4.4)^2-1=0,$$

where x is the concentration of total Ca and y is that of Ca^{++}. Hereafter in this report, the "abnormal" pattern of serum calcium means that a pair of total Ca and Ca^{++} is outside of the mentioned ellipse, or, in other words, that the substitution of the numerical values of x and y gives a positive value to the left side of the above equation.

Results with Stomach Cancer Cases

The serum calcium figures in the stomach cancer cases are shown in Table I with those of the control cases. A characteristic of the cancer cases was a lowering of I.Q. and in this point they differed from the ulcer cases, though a decrease in total Ca and in Ca^{++} was observed to be common to these two groups. This suggests that the decrease in the ionized calcium in stomach cancer is due to a depressed dissociation in addition to a depletion of total serum calcium, while that is due only to a loss of serum calcium in the case of peptic ulcer.

Abnormal patterns of serum calcium was found in almost 90 per cent of the cancer cases, in significantly higher rate than in the ulcer cases.

TABLE I. Serum Calcium Content in Stomach Cancer and in Controls

Cases	No. of total cases	Mean values*			No. of cases with abnormal patterns
		Total Ca (mg/*dl*)	Ca^{++} (mg/*dl*)	I.Q.	
Healthy adults	20	9.6 ± 0.3	4.4 ± 0.2	0.46 ± 0.01	0
Peptic ulcer	23	8.3 ± 0.4	3.9 ± 0.5	0.47 ± 0.06	18 (78.3%)
Stomach cancer	68	7.9 ± 0.2	3.1 ± 0.3	0.38 ± 0.03	61 (89.6%)

* With 95 per cent confidence ranges.

Relation with the extent of cancer progress

The concentration of total Ca and Ca^{++} in the stomach cancer cases are shown in Table II for various extent of the cancer spreads. Both the concentrations, especially that of Ca^{++}, were significantly low and the rate of occurrence of the abnormal patterns of serum calcium was extremely high in cases with the progressed disseminations or with the widespread metastasis, and consequently in those at the advanced stage of cancer progress.

TABLE II. Relation of Serum Calcium Figures in Stomach Cancer with Extent of Cancer Progress

Cancer spreads*		No. of total cases	Mean values (mg/dl)		No. of cases with abnormal patterns
			Total Ca	Ca^{++}	
Peritoneal dissemination	P.C. 0	8	8.4±0.6	3.9±0.9	5 (62.5%)
	I	24	8.1±0.3	3.0±0.4	22 (91.7%)
	II	29	7.8±0.3	3.0±0.4	27 (93.1%)
	III	7	7.6±0.7	2.4±0.4	7 (100.0%)
Lymph-nodal involvement	L.M. 0	6	8.2±0.8	3.7±0.6	3 (50.0%)
	I	10	8.1±0.7	3.5±0.7	8 (80.0%)
	II, III	47	7.9±0.5	2.8±0.3	45 (95.7%)
Stage of progress	Stage I	11	8.3±0.4	3.6±0.4	7 (63.3%)
	II	48	7.9±0.2	2.9±0.4	45 (93.7%)
	III	9	7.5±0.7	2.5±0.5	9 (100.0%)

* For explanations, see Table II of the First Report.[4]

Relation with morphology of the main tumor (Table III)

Though Ca^{++} content was comparatively low in cases with large tumors,

TABLE III. Relation of Serum Calcium Figures in Stomach Cancer with Morphology of the Tumor*

Morphology†		No. of total cases	Mean values (mg/dl)		No. of cases with abnormal patterns
			Total Ca	Ca^{++}	
Size	Small	16	8.1±0.4	3.2±0.4	13 (81.2%)
	Medium	16	7.9±0.4	3.2±0.3	16 (100.0%)
	Large	27	8.0±0.3	2.8±0.4	23 (85.2%)
Gross appearance	Circumscribed	47	8.0±0.2	3.1±0.3	42 (89.4%)
	Infiltrative	12	7.9±0.5	3.0±0.8	10 (83.3%)
Histological feature	Adenomatosum	27	7.9±0.2	3.2±0.4	23 (85.2%)
	Solidum	28	8.0±0.2	3.0±0.4	25 (89.3%)
	Mixed	4			

* In 59 cases subjected to gastrectomy.

† Cf. Table III of the First Report.[4]

such a close relation as in the case of the serum protein figures[5] and the red cell pictures[6] was not observed between the serum calcium figures and the size of the tumor. The serum calcium figures were, on the other hand, found to depend neither upon the gross features nor upon the histological views of the main tumor.

SUMMARY

Serum calcium figures in stomach cancer were studied with special interest to their relation with the morphological aspects of the lesion, taking ionization of serum calcium into consideration.

1) The concentration of serum calcium, especially that of the ionized calcium, was generally low in stomach cancer, and significantly lower even when compared with peptic ulcer cases. The abnormal patterns of serum calcium, defined from both the concentration and the extent of dissociation, were found in 89.6 per cent of the stomach cancer cases.

2) The serum calcium figures in stomach cancer were closely dependent upon the extent of the cancer spreads that was revealed by operations, a little upon the tumor size, but wholly not upon the gross nor microscopic features of the tumor.

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Tohoku J. Exper. Med., 1960, 73, 31-36

Blood Pictures in Stomach Cancer, Especially on Their Relation with Cancer Progress

Fourth Report: Supplement and Conclusions

By

Iwao Yamaguchi, Kazuhiko Inoue, Yasushi Nishiyama, Tsunemasa Suzuki, Tetsuo Yoshinari, Shozo Okuguchi, Ryoichi Motoki and **Koji Onuma**

From Prof. M. Muto's Surgical Clinic, School of Medicine, Tohoku University, Sendai

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In the preceding papers of this series[1)-3)] we have reported some of the changes found in the blood constituents of stomach cancer cases, especially on their relation with the progress of the lesion. Here will be presented the results of an additional experiment, with the conclusions for the whole series.

The experimental research consisted of injecting rabbits for a long time with an extract of the tissue of stomach cancer and following the changes in their blood constituents. This work is also a sequel to our studies on the toxic substances in stomach cancer, published in this Journal.[4)-7)]

EXPERIMENTAL

Method

Animals

The animals used in this work were 15 adult male rabbits weighing 2.2 to 2.6 kg. They were kept separately and fed on a definite diet (100 g. of a 4 : 4 : 1 : 1 mixture of barley, wheat, soya bean cake and dried fish powder and 300 g. of green vegetables, per animal per day).

Materials and Method for Injection

Materials. Fresh tumor tissues were obtained from the stomachs immediately after operation, washed to remove the blood and mucus, and then homogenized. To the homogenate was added, while thorough mixing, 10 times as much (by weight) physiological saline containing sodium ethyl-mercurithiosalicylate (as a preservative) at 1 : 10,000, yielding the cancer extract, the supernatant of which was used for the injection.

The control materials were the following two: the stomach mucosa extract

山口　巌，井上和彦，西山　安，鈴木常正，吉城哲夫，奥口昭三，元木良一，大沼弘治

prepared from mucous membrane of the stomach by a procedure similar to above, and the plain solvent, i.e. physiological saline containing the preservative at the mentioned concentration.

Method. Five rabbits were used for each of the three materials (group I for the cancer extract, group II for the mucosa extract, and group III for the plain solvent). They were injected subcutaneously at the back with 2.0 cc. of the material, every third day in a duration of 103 days, 35 times in total.

Method for Measurement

The subjects followed were the concentration and the electrophoretic pattern of serum protein, nephelogram of the serum, red cell pictures and the serum calcium content. These were studied every six days, in the same manner as in the preceding reports.

Results

Serum Proteins

Before injection of the materials, the mean values in the rabbits were 5.8±0.3 g/d*l* for total protein (T.P.), 3.1±0.2 g/d*l* for albumin (Al.), 0.9±0.1 g/d*l* for α-globulin (α-Gl.), 1.0±0.1 g/d*l* for β-globulin (β-Gl.), 0.8±0.1 g/d*l* for γ-globulin (γ-Gl.) and 1.14±0.07 for the albumin-globulin ratio (A/G). Their changes during the successive injections are shown in Table I.

A moderate decrease in α-Gl. and a remarkable increase in γ-Gl. were observed both in the groups I and II, possibly as a reaction against the foreign tissue

TABLE I. Changes in Serum Proteins*

Days† →		13	25	37	49	61	73	85	79
Group I (Cancer extract)	T.P.	+0.11	+0.09	+0.10	+0.08	+0.10	+0.07	+0.03	+0.03
	Al.	+0.11	+0.03	−0.02	0.00	−0.01	−0.07	−0.08	−0.11
	α-Gl.	−0.10	−0·28	−0.12	−0.19	−0.15	−0.24	−0.32	−0.30
	β-Gl.	+0.04	+0.23	−0.06	+0.15	+0.14	+0.10	+0.09	+0.15
	γ-Gl.	+0.56	+0.81	+1.02	+0.83	+1.00	+1.27	+1.11	+1.10
	A/G	0.00	−0.11	−0.22	−0.15	−0.20	−0.25	−0.21	−0.26
Group II (Mucosa extract)	T.P.	+0.07	+0.09	+0.05	+0.09	+0.06	+0.02	−0.11	+0.05
	Al.	+0.04	+0.06	+0.03	+0.08	+0.08	+0.14	+0.07	+0.14
	α-Gl.	+0.11	+0.04	−0.02	+0.01	−0.07	−0.21	−0.21	−0.11
	β-Gl.	+0.06	+0.16	+0.10	+0.13	+0.05	−0.09	−0.11	−0.07
	γ-Gl.	+0.16	+0.23	+0.19	+0.24	+0.21	+0.18	+0.09	+0.11
	A/G	−0.04	−0.06	−0.05	−0.02	+0.08	+0.26	+0.20	+0.21
Group III (Plain solvent)	T.P.	+0.01	+0.02	−0.02	+0.01	−0.02	−0.04	−0.02	0.00
	Al.	+0.08	+0.03	+0.01	+0.01	0.00	−0.03	+0.03	+0.02
	α-Gl.	+0.11	+0.10	−0.03	+0.02	−0.05	−0.16	−0.18	−0.04
	β-Gl.	−0.04	−0.02	−0.01	+0.01	−0.01	+0.03	−0.01	−0.02
	γ-Gl.	−0.03	0.00	−0.02	−0.02	0.00	0.00	−0.04	+0.03
	A/G	+0.07	+0.01	+0.05	+0.02	+0.04	+0.04	+0.11	+0.05

* Average rate of increase or decrease to the value before the first injection.

† After the first injection.

materials injected. Al. and A/G were, however, decreased gradually in the group I rabbits, as in the case of stomach cancer patient,[1] though these were rather increased in the group II. Each value showed, on the other hand, only little fluctuation in the group III.

Nephelogram

The serum nephelograms of the rabbits were somewhat different from those in the healthy beings,[1] showing smaller b (ranging from 8 to 11), larger T (7 or 8) and smaller W (5 to 7).

Changes in b, T, and W-values during the long-term injections are shown in Table II. In the group I, T was increased significantly and b and W were both remarkably decreased, resulting in the widening and the shift to the left of the curve which were similar to the findings in stomach cancer cases,[1] though only a moderate decrease of T, that is, the widening of the curve, was observed in the group II. The changes were within the physiological fluctuations in the group III.

Table II. Changes in Nephelographic Findings of the Serum*

Days →		19	31	43	55	67	79	91	103
Group I (Cancer extract)	b	−0.26	−0.34	−0.51	−0.35	−0.51	−0.53	−0.43	−0.47
	T	+0.20	+0.44	+0.67	+0.74	+0.77	+0.90	+0.87	+0.88
	W	−0.37	−0.78	(decreased to 0)					
Group II (Mucosa extract)	b	+0.05	−0.04	−0.01	−0.04	−0.04	−0.06	−0.07	−0.06
	T	+0.11	+0.19	+0.22	+0.23	+0.30	+0.22	+0.22	+0.28
	W	−0.06	−0.09	−0.05	−0.02	−0.11	−0.03	+0.05	−0.03
Group III (Plain solvent)	b	−0.02	0.00	0.00	−0.01	+0.03	−0.02	−0.04	−0.02
	T	+0.11	+0.03	+0.14	+0.03	+0.06	+0.06	+0.06	−0.08
	W	−0.10	−0.06	−0.06	−0.02	−0.03	−0.06	−0.02	−0.04

* Meanings of the figures are the same as in Table I.

Red Cells

The mean values of the red cell figures of the untreated rabbits were as follows: reticulocytes (Ret.), 37±8 per a thousand red cells; red cells (RC), 6,030,000±330,000 per mm^3. of blood; hemoglobin concentration (Hb), 12.2±0.5 g/dl.; mean corpuscular volume (MCV), 67.5±2.5 μ^3; mean corpuscular hemoglobin (MCH), 20.4±0.9 $\gamma\gamma$.

Their changes with the successive injections are given in Table III. In the rabbits injected with the cancer extract (group I), RC and Hb were gradually decreased in parallel to each other, accompanied with the marked diminution of Ret. suggestive of the hematopoietic depression. In these cases, a slight increase in MCV was also observed.

TABLE III. Changes in Red Cell Pictures*

Days →		19	31	43	55	67	79	91	103
Group I (Cancer extract)	Ret.	+0.23	−0.29	−0.54	−0.39	−0.52	−0.38	−0.41	−0.37
	RC	+0.01	−0.02	−0.05	−0.05	−0.12	−0.16	−0.14	−0.15
	Hb	+0.03	+0.02	−0.02	−0.01	−0.04	−0.13	−0.13	−0.15
	MCV	+0.08	+0.06	+0.05	+0.09	+0.08	+0.10	+0.11	+0.11
	MCH	+0.03	+0.04	+0.03	+0.05	+0.05	+0.03	+0.02	0.00
Group II (Mucosa extract)	Ret.	+0.47	+0.42	+0.30	+0.16	+0.16	+0.39	+0.22	+0.21
	RC	−0.05	−0.09	+0.03	+0.03	+0.01	−0.01	+0.03	+0.02
	Hb	−0.01	+0.06	+0.01	+0.01	+0.04	−0.01	+0.09	−0.01
	MCV	+0.12	+0.15	+0.06	−0.02	−0.02	+0.02	0.00	+0.01
	MCH	−0.05	+0.17	−0.01	−0.04	+0.03	−0.01	+0.05	−0.03
Group III (Plain solvent)	Ret.	+0.13	+0.14	+0.17	+0.13	+0.17	+0.09	+0.10	+0.09
	RC	−0.01	−0.04	−0.01	−0.01	−0.04	0.00	0.00	0.00
	Hb	+0.05	+0.01	+0.05	+0.05	−0.01	+0.02	+0.08	+0.04
	MCV	+0.02	+0.02	0.00	0.00	+0.01	+0.04	−0.02	0.00
	MCH	+0.07	+0.05	+0.06	+0.05	+0.02	+0.03	+0.08	+0.04

* Meanings of the figures are the same as in Table I.

TABLE IV. Changes in Serum Calcium Content*

Days →		19	31	43	55	67	79	91	103
Group I (Cancer extract)	Total Ca	−0.14	−0.05	−0.03	−0.01	−0.04	−0.05	−0.03	−0.03
	I.Q.	−0.03	−0.10	−0.18	−0.18	−0.07	−0.22	−0.36	−0.35
Group II (Mucosa extract)	Total Ca	−0.07	−0.04	0.00	−0.10	+0.01	+0.18	+0.07	−0.04
	I.Q.	+0.01	−0.05	−0.02	+0.01	+0.02	+0.02	+0.05	+0.06
Group III (Plain solvent)	Total Ca	+0.16	+0.14	−0.03	+0.03	+0.01	+0.13	−0.02	0.00
	I.Q.	+0.02	+0.01	−0.06	−0.01	+0.01	+0.01	+0.02	+0.04

* Meanings of the figures are the same as in Table I.

In the other groups, the changes in the red cell figures were insignificant, except that Ret. was increased markedly in the group II and moderately in the group III, especially at the early stage of the injections.

Serum Calcium

Before the injections, the total concentration and the ionization quotient (I. Q.), i.e. the ratio of an ionized fraction to the total, of serum calcium were 11.6±0.7 mg d*l* and 0.47±0.04, respectively on an average. As shown in Table IV, I.Q. of the group I became gradually lower with succession of the injections, to show a similarity to the case of human stomach cancer cases,[3] while those of the groups II and III were within the physiological ranges. The total concentration of serum calcium, on the other hand, remained almost unchanged, in each of the groups.

DISCUSSION AND CONCLUSIONS

Cases with stomach cancer show, in general, various changes in the blood constituents, though their extents are much different from case to case. The present studies have been undertaken to find the relation between the hematological condition in stomach cancer and the morphological aspects of the lesion which are revealed by surgical explorations at operation and the pathological examinations on the resected stomach.

In answer to this, the results hitherto presented have indicated that the following two are the most significant factors of the lesion on which the extent of the blood changes is closely dependent: one is the extent of "progress" of the lesion including the peritoneal dissemination, lymph-nodal involvement and metastasis to other organs, and the other is the size of the tumor. Gross or microscopic features of the main tumor were, contrary to their importance in pathology, found to be little concerned with the present matter.

The same relations as above were observed previously as to the depressed liver catalase activity in stomach cancer,[8] and it is presumable that most of the general patho-physiological changes in stomach cancer may show similar relations. It is of great interest, in addition, that the mentioned relations have a strong resemblance to those between the post-operative prognosis in stomach cancer and the findings of the lesion at operation.[9]

The mechanism of such hematological changes in stomach cancer may be very complicated, but this is beyond the subject of this article. We could observe, however, in a model experiment which consisted of injecting the rabbit successively for a long time with a tissue extract of stomach cancer, gradual changes in the blood constituents, some of which resembled those of the progressed stomach cancer cases. This may be said to suggest that the action of toxic substances contained in the cancer tissue is possibly one of the factors causing the hematological changes in stomach cancer.

SUMMARY

1. Rabbits were injected with (1) a saline extract of the stomach cancer tissue, (2) a saline extract of the normal gastric mucosa and (3) the plain solvent, respectively, successively for 103 days, changes in their blood constituents being studied in relation to the clinical observations in the preceding reports.

2. A brief discussion was made on the nature of the hematological changes in stomach cancer, particularly on their relation with morphological aspects of the lesion, at the conclusion of the series.

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A Study on the Sensitivity of the Target Organ to Female Sex Hormones

By

Masakuni Suzuki, Yuzo Murakami, Tetsuro Abe and Ichiro Kumagai

From the Department of Obstetrics and Gynecology, Tohoku University School of Medicine, Sendai; Director : Prof. K. Kushima

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Tests measuring of hormones have been undertaken and dosages of hormone preparations have been applied, in diagnosing and treating endocrine disorders. It must be pointed out, however, when a hormone manifests a certain effect, it is not only the amount of the secreted hormone, but also the reaction of the target organ subject to the effect to the hormone. That is, the level of sensitivity of the organ concerns the efficacy of the hormone. Seeing that in the current practice, this problem of the sensitivity of the target organ has been nearly neglected, we made an attempt to inquire into this problem.

As materials, we took up the uterus and its endometrium as the organs known to be most sensitive in the reaction to female sex hormones.

Weight of the Uterus of Mice

Experimental Materials and Method

Young mice of three weeks after birth were devided into four groups of five or six heads each, as follows : 1) A group of mice injected intramuscularly on the back once a day for 7 consecutive days with 7 γ of diphosphopyridine nucleotide (DPN hereunder) dissolved in 0.1 ml of distilled water. 2) A group of mice similarly injected for 7 days with 4 γ of estradiol dissolved in 0.1 ml of olive oil. 3) A group given the above two injections in combination for 7 days. 4) A control group injected with 0.1 ml of blank olive oil daily for 7 days. All the animals were sacrificed by decapitation 24 hours after the last injection, i.e. on the 28th day after birth and the wet weight of the extracted uterus was measured with a torsion balance.

Experimental Results

The results obtained are shown in Fig. 1. The weight of the fresh uterus was slightly larger in the DPN group (13.0 mg on the average) and notably larger in the estradiol group (31.7 mg) than in the control group (averaging 10.6 mg).

鈴木雅洲，村上裕三，安部徹良，熊谷一郎

Fig. 1. Wet-weight of the mouse uterus after administration of estrogen as well as DPN.

Thus, in the estradiol-injected animals, the weight of the uterus rose to about thrice that in the control animals on the average; in the former, the uterus was macroscopically frankly blood-congested and edematous. In the DPN plus estradiol group, the average uterine weight reached 38.7 mg and the uterine changes were also the most prominet.

ATP in the Human Endometrium

Experimental Materials and Method

The endometrium of four women with normal uterus in the proliferative phase, 1 in the ovulation phase, 3 in the secretory phase with normal menstrual cycle, 1 in the proliferative phase and 5 in the secretory phase with functional bleeding were sampled for measurement of the ATP.

The specimens were sampled by curettage, immediately frozen with dry ice, weighed with a torsion balance, mixed with 5 ml of 0.6 N cold perchloric acid (PCA) and homogenized. After centrifuging, the supernatant was again subjected to extraction with the same volume of 0.2 N PCA. The pH value of the both extracts was lowered to 7.0 with 5 N KOH while keeping them cool and the produced $KC10_4$ was removed by centrifuging. The supernatant fluid thus obtained was analysed by column, ion-exchange chromatography. The absorption layer consisted of "Dowex 1 × 10 anion-exchange resin" of Dow Chemical Co. Ltd. (U.S.A.). Before the test, the column was repeatedly perfused with H_2O, 1 N NaOH, H_2O, 1 N HCl and H_2O always in the same order for activating the resin. The A eluent solution was 0.01 M NH_4Cl, the B solution : 0.003 M HCl, the C solution : 0.01 M HCl + 0.2 M NaCl, and the D solution : 0.2 M HCl + 0.2 M NaCl and stepwise elution analysis was carried out with these. The ultra-violet absorption E_{260} was measured with an electric spectro-photometer using H_2O as blank and the quantity of the fraction was determined by the following formula using the molecular absorption coefficient obtained.

$$C = \frac{E}{B \cdot I}$$

C : Concentration E : Extinction
I : Thickness of the solution measured

According to Volkin and Cohn, the molecular absorption coefficient of adenosine at the maximum absorption value at pH 2 with H_2O as blank (B) is 14,200 (258 mμ).

Experimental Results

The mean measured values of AMP, ADP and ATP of the human endometrium are shown in Fig. 2. The specimens from the patients with functional bleeding were divided into the groups of those from cases in the proliferative and the secretory phase by their histological findings. In the figure, the values are represented by black bars. The white bars indicate the mean values obtained with five cases in the proliferative phase and three in the secretory phase of women with normal menstrual cycle, and the black bars show those obtained with five cases in the proliferative phase and one in the secretory phase of women with functional bleeding.

The AMP value is low in all the cases. The ADP and the ATP values were

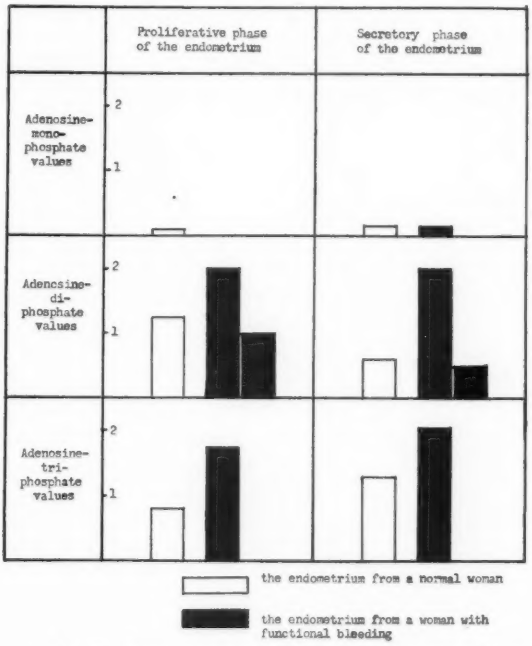

Fig. 2. Mean values of adenosine-phosphate of three cases of the human endometrium.

both found markedly higher in the cases with functional bleeding in the secretory as well as the proliferative phase.

Acetylcholine in the Human Endometrium

Experimental Materials and Method

Specimens of human endometria sampled directly upon hysterectomy and upon curettage were used. These were cleansed of blood with cooled physiological saline directly after sampling and stored in an electric refrigerator not longer than 24 hours before measurement of their acetylcholine.

The method of Feldberg[1] was followed for making extracts from the specimens. The process consisted of homogenising a specimen in 2 ml of N/3 HCl, heating at 80°C for one hour, neutralizing with N/3 NaOH after cooling and mixing with frog-Ringer solution to the volume so that 1 ml of the mixture contained 1 mg of the endometrial tissue.

A lung of toad was suspended in a Magnus vat containing 30 ml of frog-Ringer solution and its contraction was recorded with a kymographion.[2] The

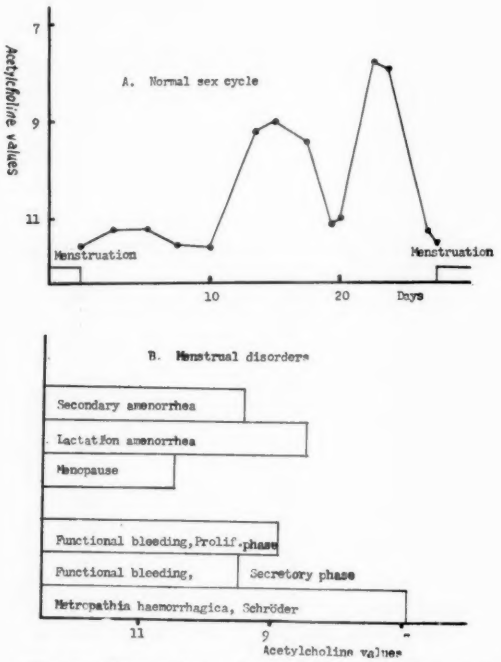

Fig. 3. Mean values of acetylcholine of five cases of the human uterine endometrium.

contraction of the toad lung when the specimen extract was added to the solution was measured and the acetylcholine content in it was estimated by comparison of this measured value with the contraction of the lung when 10^{-15} g/ml to 10^{-5} g/ml of acetylcholine was added to the Ringer solution in the vat. The frog-Ringer solution used contained 6.0 g of NaCl, 0.075 g of KCl, 0.01 g of $CaCl_2$, and the sensitizers $CaCl_2$ (0.2 g), $BaCl_2$ (0.01 g) and caffeine (0.01 g) dissolved in 1,000 ml of distilled water. Its pH value was kept within the range of 6.4–6.8.

Experimental Results

The measured values of the endometrial acetylcholine content were as shown in Fig. 3. The figures show the values of the quantity of acetylcholine chloride (Ovisot) equivalent to 1 g of fresh wet endometrium in negative logarithms of the acetylcholine concentration.

The mean measured value obtained from five normal women with normal menstrual cycle is also given in Fig. 3, suggesting that the acetylcholine value in

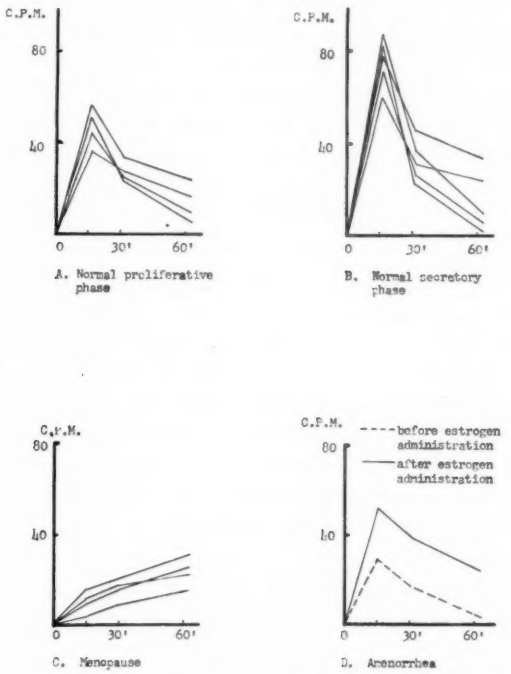

Fig. 4. The count per 1 ml of blood after the infusion of ^{32}P into the uterine cavity.

the endometrium changes rather widely with the menstrual cycle. In the cases with functional bleeding and other menstrual disorders, the value showed more perceptible differences than in the normal cases.

^{32}P-Uptake of the Human Endometrium

Experiental Materials and Method

0.1 ml each of sodium phosphate solution labeled with ^{32}P was introduced into the uterine cavity of subjects with normal menstrual cycle, and in menopause and amenorrheic subjects. Then 1 ml of blood was sampled from their cubital vein 15, 30 and 60 minutes thereafter for measuring the radioactivity with a Geiger-Müller tube.

Experimental Results

The results were as shown in Fig. 4. The ordinate shows the counts per minute per 1 ml of whole blood.

The measured values obtained with 4 cases in normal proliferative phase, 4 cases in normal secretory phase, 4 cases after menopause and 1 amenorrheic subject were studied in comparison. In normal subjects, the maximum value was attained in 15 minutes, the peak being the higher in the cases in the secretory phase than those in the proliferative phase. A retrogression of the peak was observed in the cases after menopause and the curve returned to the form of a normal proliferative phase in the amenorrheic case after injection of 50,000 units of estrogen once a day for 3 successive days.

Tissue Respiration of the Human Endometrium

Experimental Materials and Method

Endometrial specimens were sampled from subjects with normal menstrual cycles and with functional bleeding, immediately immersed in 0.9 per cent KCl solution and the blood and the surplus water were drawn off with filter paper. 100 mg of each specimen was sliced and subjected to the measurement within 30 minutes. 1 ml of Krebs-Ringer-phosphate buffer (pH 7.4) was used to incubate the sliced specimens. The respiration values were measured with a Warburg's manometer and recorded as the Warburg's homogenous metabolism coefficients Qo_2. A piece of filter paper soaked with 0.2 ml of 20 per cent KOH solution was placed in the by-chamber of the manometer.

Experiental Results

The measured results are shown in Fig. 5. The Qo_2 values were found to fluctuate with the menstrual cycle, but were always very perceptibly lower in the subjects after menopause and markedly risen in the subjects with functional bleeding. When 0.1 γ/ml of estrogen was added to the buffer, the Qo_2 was found risen in the normal specimens in the histologically proliferative phase but in-

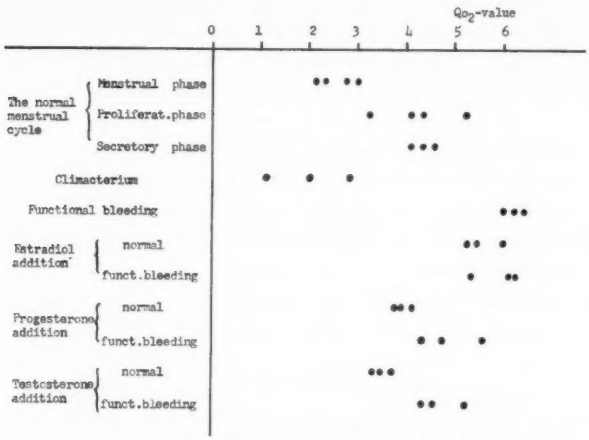

Fig. 5. Changes of endometrial Qo_2-values in the normal and abnormal mucus membranes of the uterus.

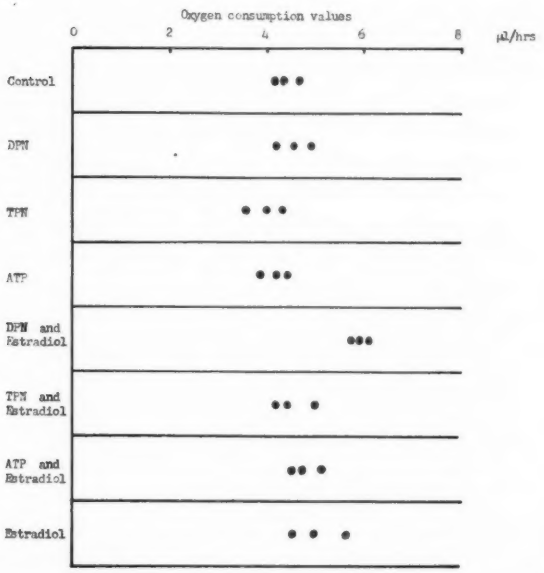

Fig. 6. Oxygen consumption values of the human uterine endometrium in the secretory phase under the influence of various Co-enzymes.

dicated weakening of the tissue respiration in the cases with functional bleeding. The reaction being quite opposite in tendency in these two kinds of specimens. When progesterone or testosterone was added, the reaction was always in the direction of depressing the tissue respiration in specimens from the normal subjects as well as those with functional bleeding.

When various co-enzymes were added, after addition of 0.75 γ/ml of DPN alone, the Qo_2 was very slightly raised in normal endometrium in the secretory phase, but not at all after addition of 0.75 γ/ml of TPN or 2.5 γ/ml of ATP. After addition of estradiol the Qo_2 value was found evidently risen in normal endometrium. When any of the above co-enzymes were added in combination to estradiol before incubation, the Qo_2 value was very highly raised when DPN was used but not in any other combination.

DISCUSSION

The tissue respiration of the normal endometrium fluctuates with the menstrual cycle and at monopause, and such a change in the value seems to be due to an influence of the sex hormones.[3][4][5] In a macroscopically thickened endometrium in functional bleeding showing a histological picture of being in the proliferative phase, the Qo_2 value was found the highest among all the specimens. After the addition of estradiol, the change in the Qo_2 value showed the quite opposite tendency in normal endometrium on proliferative phase to that in the endometrium of functional bleeding. Thus, it was established that the sensitivity to sex hormones is different in an anomalous endometrium suffering from, say, functional bleeding, from that in a normal endometrium.

Upon comparative study of the results of acetylcholine and ATP measurements in normal endometria and in those in functional bleeding, it was found that in normal endometria in the proliferative and the secretory phases AMP was very low but ADP and ATP were higher than AMP in quantity, while in the endometria in functional bleeding, both ADP and ATP were found obviously increased above normal. Acetylcholine was found in the maximum quantity in the secretory phase and showed fluctuation with the menstrual cycle, but was found most abundant in the patients with Schröder's metropathy. The acetylcholine level in the endometrium in functional bleeding that showed the histological findings similar to those of normal endometrium in the proliferative or secretory phase was not very high and the lowest in the specimens from subjects who had passed their menopause. Our measurements of the level of endometrial phosphate metabolism using ^{32}P showed that the level was different in the proliferative and the secretory phases, but in the subjects in menopause and with amenorrhea the level was always lower than in those in either phase of the normal menstrual cycle. These findings lead to the conclusion that the general metabolism is abnormalized in the endometrium of the subjects with funcional

bleeding and other uterine anomalies. The anomaly in the sensitivity to sex hormones manifested in such abnormal endometria may be due to the anomaly in the general metabolism. It is impossible, however, to determine at this stage what biochemical reaction lies at the primary cause of the anomaly of sex-hormone sensitivity in such anomalous endometria.

Next, we tried to investigate the possibility of causing changes in the endometrial sensitivity to sex hormones by variously manipulating the endometrium. The tissue respiration is stimulated by application of estradiol and this action can be enhanced by treating the endometrium with the co-enzyme DPN, but TPN and ATP have no such a additive effect. The effect of estradiol in increasing the weight of the uterus of mice may be promoted by DPN. Such results have revealed the possibility of artificially changing the sensitivity of the endometrium. In the future, it may be expected that treatment of anomalous sensitivity of the target organs may become possible by some remedial measures.

Dalsace[6] reported on a case of a woman who had impaired sensitivity to sex hormones despite hypersecretion of estrogen. We have also recently experienced three cases of women who had no anomaly at all in the function of their ovaries and in whom normal ovulation could be demonstrated by basal body temperature (B.B.T.) measurement and estrogen and pregnanediol measurements as well as confirmation of corpus luteum by laparotomy, and showed irresponsiveness of the uterus to sex-hormone and remained amenorrheic. It may be that such cases are not rare at all; but little attention has been paid to the question in any part of the world. It seems such cases deserve more attention if we are to make efforts in promoting diagnosis of and improving therapy for hyposensitivity of the target organs to sex hormones.

SUMMARY

The uterus and such target organs to sex-hormones sometimes fall into dysfunction. The sensitivity of the target organ was analysed by determinations of the weight of the uterus of mice, of ATP in the human endometrium, of acetylcholine in the human endometrium, of ^{32}P uptake of the human endometrium and of the tissue respiration of the human endometrium. In such cases, we must not limit ourselves to hormone administration but also not neglect treatment of the organs with impaired sensitivity to sex hormones. The sensitivity of the target organ was enhanced by treating the endometrium with the co-enzyme DPN.

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Initial Reactions during Influenza A Virus Invasion into L Cells

Second Report : Growth Characteristics of Myxoviruses in Tissue Culture

By

Nakao Ishida and **Toyorō Ōsato**

Department of Bacteriology, School of Medicine, Tohoku University, Sendai; Prof. N. Ishida

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The crucial event in bacteriophage infection of host cells is the introduction of viral nucleic acid into the cell. This was first shown by Hershey and Chase[1] but indirectly. Later, the experiments carried out by Kozloff[2] visualized the events occurring during the invasion process of viral nucleic acid, in an exact manner.

With animal viruses, nucleic acids derived from several encephalitis and polio viruses were shown to infect the cell in mouse brain[3] or in tissue culture[4]. However, as far as the nucleic acid of Myxoviruses is concerned, the fact has not yet been fully proved[5] as that of bacteriophage or neurotropic viruses. Moreover, the process where virus particles can release their nucleic acid has never been visualized with animal viruses. Kozloff's experiment suggested us the analysis of the invasion process of myxovirus into tissue culture cell by testing the changes occurred outside the cell rather than inside. Though the two schools in Germany and England[6–12] have suggested the disintegration of myxovirus particles at the time of infection, these results were obtained by pursuing the chemical changes inside the cell following the invasion.

In this work, the system, PR8 virus and L cell, was employed. This system has been already known to allow the production of noninfectious hemagglutinins like the system PR8 virus and HeLa cell.

After collision of these two particles, one 80 mμ and the other 18 μ in diameter in phosphate buffered saline, agitation was continued for 2 hours at 37°C. As a result, hemagglutinins in the supernate rather increased in titer and they revealed different character from that of inoculum preparation. Furthermore, P^{32} which has been incorporated into the inoculum virus particle was found to be released in the supernatant but in different chemical nature from that found in the inoculum.

石田名香雄，大里外巻郎

The body of circumstantial evidences may support the idea that the disintegration of the virus particle occurred on the cell surface during the incubation period, though the question whether this is the usual step of invasion process has not been answered.

MATERIALS AND METHODS

The virus

The PR8 strain of influenza A virus was used. The strain has undergone seven passages in ferrets, 593 in mice and 170 passages in embryonated eggs. This egg line virus again received 40 to 41 passages in mice and finally propagated in embryonated eggs for obtaining concentrated inoculum to use in tissue cultures.

Labelling of the virus

Labelling of this virus was performed in a Maitland type tissue culture[13], using chorioallantoic membranes of 15-day-old embryonated hen's egg as the host tissue. All phosphorus was removed from Hanks' solution used for the medium and P^{32} in a form of orthophosphate was added in a concentration of 87 μc/ml. Allantoic fluid virus was used as the inoculum in 10^{-2} dilution. After 24 hrs. of incubation, the filtrate containing around 1,024 hemagglutinin units/ml was obtained. From this filtrate, the virus was purified by means of 2 cycle run of adsorption to and elution from chicken red cells and ultracentrifugation followed by dialysis. Technical details to do this have already appeared.[13]

Determination of radioactivity

Radiophosphorus in the form of orthophosphate in diluted HCl supplied from the Radiochemical Center, Amersham, England was used in this experiment. All determinations of radioactivity were carried out as a rule in duplicate by evaporating 0.5 ml of the preparations on planchets and counting the activity with a mica end-window Geiger-Müller tube. The results are expressed in the text as counts per minute (cpm) corrected for 1 ml amount.

Host cells

L cells were used throughout the work. They were maintained in this laboratory[14] for these 4 years in lactoalbumin-yeast extract medium containing 20% calf serum.

Hemagglutinin titrations

Virus titration was made only by hemagglutination test. Salk's pattern method was chiefly employed, but the 2 fold dilution was started from different original dilution, as has been recommended by Horsfall and Tamm[15]. Thus fairly exact titer was obtained with each specimen. Both chicken and guinea pig red cells were used to meet the possibility in detecting different hemagglutinin

(HA) characters[16].

EXPERIMENTAL

Procedures to obtain the starting materials which were designated as "L-SUP" and "INOC"

As has been described in the section of materials and methods, P^{32} labelled virus preparation was obtained in a Maitland type tissue culture. After the purification, a phsophate buffered saline suspension of the virus, which had the titer of 310 hemagglutinin units and 1,970cpm per ml and giving the specific activity of 6.35 in a sense of cpm/HA was obtained. This suspension was not contaminated more than 5% of non-viral P^{32}[13]. Then 4 bottle culture harvests of L cells, obtained after 7 days of incubation were trypsinized and washed with M/100 phosphate buffered saline of pH 7.0 (PBS). They were packed by sedimentation and 7.0×10^7 cells were suspended into 5 ml of labelled virus suspension as described above. In this condition, the ratio virus/cell was around 70.

The tube was then incubated at 37°C for 2 hours with constant agitation. The control tube containing only the inoculum virus suspended in the same PBS was treated just in the same manner. Then these two tubes were spun at 2,000 rpm for 5 minutes to remove the L cell of the first tube. The supernatant thus obtained from the first tube will be referred as *L-SUP* in the text, whereas the supernate of the second tube, i.e. the inoculum virus which was incubated at 37°C for 2 hours will be referred as *INOC*. All analyses in the text were performed simultaneously with these 2 fractions, i.e. *L-SUP* and *INOC*.

Different character of hemagglutinins found in L-SUP from that in INOC

To see the difference of *L-SUP* from *INOC*, HA assay was firstly made with both chicken and guinea pig red cells. P^{32} counts were also made with these 2 materials.

Obtained results were illustrated by Table I, where one can find unexpectedly large amount of HA in the *L-SUP*. This was particularly the case when this

Table I. Increase of HA in the L-supernatant

Sample	HA/ml*		cpm/ml†	cpm/HA	
	chicken	guinea pig		chicken	guinea pig
Inoculum	310	512	1970	6.35	3.85
L-supernatant	380	1510	1430	3.76	0.95

* The hemagglutinin titers are expressed as the reciprocal of the limiting dilution, agglutinating 0.5% chicken and guinea pig erythrocytes.

† The radioactivity is expressed as counts per minutes (cpm), corrected for 1 ml volume.

fraction was assayed by guinea pig cells. Fifty per cent decrease, which is usually the case, was not detectable with *L-SUP*, but rather the titer increased as much as 3 times of the *INOC*, which was the starting material to obtain the fraction, *L-SUP*.

In spite of this increase in HA titer, around 25% (540/1,970) of the radioactivity disappeared from this supernatant. Both 4th and 5th columns of Table I were prepared to describe the above difference, more apparently. To explain this result, disintegration of virus particles at the time of collision or invasion to the host cell will be most plausible. Hoyle[16] and Henle[17] have already proved the increase of small HA particles which agglutinate guinea pig red cells more easily than chicken red cells after the decomposition of elementary bodies with ether. Probably the same phenomenon occurred when the virus particle here used was in contact to L cell for 2 hours at 37°C.

The disappearance of radioactivity from *L-SUP* has to be explained by its adsorption to the L cell debris. The fact was later proved in a separate experiment.[18] Whether this is because of introduction of viral nucleic acid into the L cell, however, is not known.

The nature of radioactivity and HA found in the L-SUP

To approve the former results, the second experiment was conducted. Firstly, both *INOC* and *L-SUP* were treated with specific adsorbent of chicken red cells, and the count and HA left in the supernatant was compared. Secondly, the molecular size of HA as well as the radioactivity in the *L-SUP* was examined in comparison with those in *INOC* in the field of ultracentrifugation. Table II shows the results of three different experiments, where the *INOC* was always served as the control and examined in the same manner as *L-SUP*, simultaneously.

Table II. Characteristics of Radioactivity and HA Found in *L-SUP* when Compared to Those in *INOC*

Sample	Supernatant after adsorption to conc. chicken red cells* (same cpm)†		Supernatant after span at 26,000 g for 1 hr. (same HA)‡		Supernatant after span at 26,000 g for 1 hr. (same cpm)†	
	cpm	HA	cpm	HA	cpm	HA
Inoculum	74	<4 (chicken) <4 (guinea pig)	104	<4 (chicken) <4 (guinea pig)	84	<4 (chicken) <4 (guinea pig)
L-supernatnat	140	<4 (chicken) <4 (guinea pig)	246	80 (chicken) 80 (guinea pig)	284	80 (chicken) 80 (guinea pig)

* 3 ml of 2% chicken cell suspension are packed and mixed with 1 ml of each tested material. Adsorption was conducted at 0°C for 90 min.

† Started from samples possessing the same radioactivity of 1430.

‡ Started from samples possessing the same HA titer of 310.

In the first column, one can see that hemagglutinins both in *L-SUP* and *INOC* are all adsorbed to chicken red cells. Whereas, around 2 times radioactivity was found in the *L-SUP* when compared to that in *INOC*.

In this case, both materials were examined after adjusting to be the same radioactivity of 1,430. As the count found in the *INOC* should be understood as the contaminated inorganic or organic P^{32} of the starting material, the chemical nature of the increment found in *L-SUP* when compared to *INOC* (140−74=66) is rather to be taken into account. The fact may at least suggest that some fraction of P^{32} found in *L-SUP* is not bound to virus particles.

In both second and third columns, HA and radioactivity left in the supernatant after the centrifugation of both *L-SUP* and *INOC* at 26,000 g for 1 hour were compared. The experiment, however, was done in two different manners. One run was started after adjustment of the HA titer in both *L-SUP* and *INOC* to the same titer of 310 (column 2), and the other after adjusting to be the same radioactivity of 1,430 (column 3). In both experiments, the fact was established that fairly higher percentage of HA in the *L-SUP* was not sedimentable by this centrifugation. This experiment again indicated the fact that some fraction of the radioactivity in *L-SUP* was not bound to virus particles of usual size.

Table III. Non-sedimentable Radioactivity Found after the Centrifugation at 100,000 g for 2 Hrs.

Sample	Supernatant after centrifugation at 100,000 g for 2 hrs.	
	cpm/ml	HA/ml
Inoculum	81	<4
L-supernatant	125	8

Then the effort was made to precipitate these small hemagglutinins to see the distribution of P^{32} in the *L-SUP*. Table III shows the result, where the supernatants obtained from former experiment with both *INOC* and *L-SUP* were again centrifuged at 100,000 g for 2 hours. Though 8 out of 80 HA unit was still left in this supernate of *L-SUP*, around 90% of small HA was thus removed. Still the difference of 44 cpm/ml was found between *L-SUP* and *INOC*.

In inspecting the results illustrated by Tables II and III, one can notice that cpm found in *INOC* after 3 different treatments are relatively equal. That is, when mother solution of 1,430 cmp/ml was examined for its purity one by adsorption to chicken red cell, the other after sedimentation at either 26,000 g or 100,000 g for 1 or 2 hours, 74, 84, and 81 cpm were found to be not bound to virus particles. On the basis of this figure, the difference in radioactivity between *L-SUP* and *INOC* should be considered in a careful manner.

This kind of calculation was made as follows: From 284 cpm (see Table II, column 3) found after centrifugation at 26,000 g, 84 should be substracted as the contaminant found in starting material. Then it gives the answer of 200 count which should be understood as the fraction not bound to virus particles, but released from 1,970 count of the starting material. In other words, at minimum 14% (200/1,430) of the residual count found in L-SUP was in a form different to that bound to virus particles. Among this fraction of 200 count, some fraction of 66 count (140–74, see Table II column 1) was changed into a form to be not adsorbed to red cells. Then the fraction of 134 out of 200 (200–66) should have had the activity to be adsorbed to red cells but was left in the supernatant after sedimentation at 26,000 g. On the other hand, calculation from Table III gives us an answer that at least fraction of 44 (125–81) was not sedimentable even after centrifugation at 100,000 g. Taken together, if this fraction of 44 count in a state of such small particles below 20 mμ is considered to be the same entity with the fraction calculated as 66, which is not adsorbable to chicken red cells, whole story is quite easy to understand. This was later proved through another approach.

In summary, around the three fourth of the released radioactivity in *L-SUP* had the sedimentation constant similar to small hemagglutinins. In adsorption test, also almost the same count has been proved to be adsorbable to chicken cells. Thus the most plausible explanation will be that some phospholipid involving hemagglutinin activities will be the main splitting product of this collision. However, this effect should be further examined, under the alternative assumption that purified small hemagglutinins should not be contaminated with any phosphorus.

Ether treatment of both L-SUP and INOC

The calculation described above suggested us the possibility that phospholipid of virus particles may be splitted in a form accompanying with small hemagglutinins. However, an alternative idea is that they may exist in a form of polymerized phospholipid which is adsorbable to red cell by non-specific adsorption. To get the information on these 2 possbilities, ether extractability of the radioactivity contained in both fraction was examined.

Thus both *L-SUP* and *INOC* were treated first with equal amount of ether at 27°C for 2 hours under constant shaking. The radioactivity found in ether, however, was fairly smaller than expected (see Table IV). Moreover, there was no difference between *L-SUP* and *INOC*. Then the subsequent extraction was made at 37°C for 2 hours under the same condition. This time the radioactivity in *L-SUP* was larger than that in *INOC*. When these activities, one extracted at 27°C, the other at 37°C are combined, around 100 count were obtained with *L-SUP*. However, more important answer is the difference of around 60 count

TABLE IV. Ether-soluble Fraction Detectable in Both *INOC* and *L-SUP*

Sample	cpm of ether phase		Total
	With an equal volume of ether agitated for 2 hrs. at 27°C*	Further extraction with an equal amount of ether under agitation at 37°C for 2 hrs.*	
Inoculum	27	16	43
L-supernatant	26	75	101

* Constant agitation is conducted on the shaking machine.

found between *L-SUP* and *INOC*, at the time of extraction at 37°C. The fact may suggest the easiness in separating phospholipid P from *L-SUP*. However, this fraction will not be a free phospholipid, because it was not easily extracted at 27°C.

DISCUSSION

Suspensions of both PR8 virus and L cells were incubated at 37°C for 2 hrs. and the supernatant obtained after sedimentation of the cell was employed for analyses, in an effort to detect the fate of radioactivities and hemagglutinin components derived from the inoculum particles. This was thought to be a procedure of choice in analyzing the outcome of viral invasion rather than the fractionation trial of the infected cell after their disintegration, from considerations described below.

Firstly, analysis of the aqueous solution but not cell constituents was believed to eliminate at least one of the possibilities of mislead, due to the inadequate homogenization procedure. Secondly, as influenza viruses are known to contain as low as one per cent nucleic acid, the tracing of ribonucleic acid phosphorus inside the cell was thought to be a fairly difficult matter in revealing the exactness. Rather a phospholipid entity detectable as high as 25% of the total virus weight was thought to be a better indicator in the tracer experiment using P^{32}. Moreover, this fraction has been believed to be located in the outer layer of the particle, and will be possibly disintegrated and liberated at the time of infection. Thirdly, many experiments made along the same line, suggested the absence of disintegrated hemagglutinins in the cell, except that of residual character. These series of considerations pointed out the advantages to test the chemical and physical characteristics of the radioactivity and hemagglutinins in the supernatant, obtained after the incubation of L cell with radioactive inoculum virus.

Experimental results obtained so far revealed several interesting facts. Firstly, the increase of hemagglutinin occurred after the incubation at 37°C.

This was particularly the case when the titer was tested with guinea pig cells. Secondly, some 25% of the radioactivity in the inoculum was not detectable in the supernate. Taken together, plausible explanatation will be the splitting of the inoculum virus into small hemagglutinins followed by the introduction of viral nucleic acid into the cell. But to confirm the latter possibility, another experimental run is now going on[18]. Repeated washings and RDE treatment of these cells released only a few per cent of the radioactivity found in the cell debris[18].

In the third experimental run, radioactivity found in the supernatant was characterized to find out the difference from that in the inoculum.

In the L-supernatant, one can anticipate the existence of various substances derived from inoculum particles. They will be intact viruses, smaller hemagglutinins of various size, viral phospholipid (may be partly phospholipid and partly phospholipoprotein) and possibly viral nucleoprotein. One can also imagine that they may distribute in various fraction in the field of centrifugation, owing to their grade of aggregation.

In performing the experiment, the count left in this fluid after adsorption to highly concentrated chicken cells was first examined. This entity was thought to involve a phospholipid phosphorus. Further in the field of ultracentrifugation, non-sedimentable fraction was examined. Significant amounts of HA and count still remained in the resulting supernatant even after a sufficient span to precipitate viral particles of usual size. This time, the P^{32} count found in the supernatant was thought to represent the free phospholipid and also phospholipid associated with small hemagglutinins.

Illustrated results may at least indicate the presence of various disintegrated materials in the L-supernatant. Further centrifugation at 100,000 g for 2 hrs. still left small amount of HA and 8.7% of the radioactivity in the supernatant, suggesting these disintegrated components may be fairly light and small.

In all experiments described above, detectable radioactivity after adsorption or sedimentation may exclude the contamination of P^{32} associated with virus particles of normal size. Further, there should be fairly a small amount of inorganic P^{32} in the *L-SUP*, because of the possibility to be adsorbed to L cells, during 2 hrs incubation.

Finally, the possibility to transfer these phospholipid contained in both *INOC* and *L-SUP* to ether was examined. The result obtained at 27°C indicated no difference between the *INOC* and *L-SUP* ,while at 37°C highly increased count was recognized with the *L-SUP*. This may be due to easiness in removing viral phospholipid in the *L-SUP* after their disintegration. From this result, it may be suggested that on infection, proteolytic enzyme of the cell will play a role in splitting viral surface phospholipoprotein. Though the experimental results

were not involved here, when the chicken red cells were used as the counterpart of L cells, such a remarkable change of the radioactivity derived from the inoculum was never found.

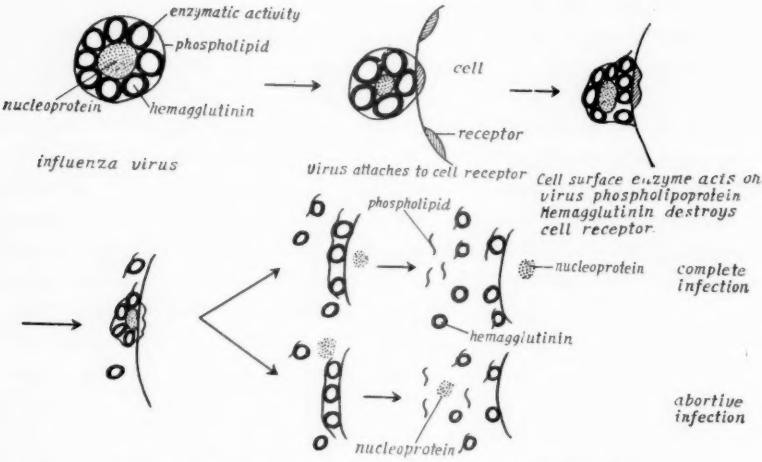

Fig. 1. Scheme of Influenza Virus Invasion to Host Cell.

Thus, all the results described in this paper indicate that influenza virus may disintegrate on infection. Actual calculations of radioactivity in anticipating the nature of these sources were already described in the text.

On the basis of this observation, the initial stage of viral infection was characterized and schematically illustrated by Fig. 1.

Firstly, the virus attached to the host cell receptor. Secondly, cell surface enzymes acted on the coat of virus. On the other hand, a part of virus hemagglutinins destroyed cell receptor and were eluted into medium from cell surface. However, a part of them may remain on the surface. In this situation, virus particles were disintegrated. Destroyed viral phospholipid was also released into medium or remained on the cell surface[18]. Finally, viral ribonucleoprotein may penetrate into host cells. However, there may be a possibility that the ribonucleoprotein or ribonucleic acid will be ejected into medium as abortive infection[18].

SUMMARY

The initial stage of virus-cell interaction was studied in a system of PR8 virus and Earle's L cell. Used virus was highly labelled with P^{32} and not contaminated more than 5% of P^{32} other than virus.

In conducting the experiment, these 2 particles, i.e. virus and host cell were

combined and agitated for 2 hrs. at 37°C. Then the cells were removed from the mixture by centrifugation and the nature of hemagglutinins and distribution of radioactivity in this supernatant were compared to those in the inoculum.

Hemagglutinin titer left in the supernatant has been proved to increase after the incubation, particularly when tested with guinea pig red cells. Furthermore, the P^{32} count detectable after red cell adsorption or after ultracentrifugation which will be enough to sediment elementary bodies, has been increased. Part of them was sedimented with small hemagglutinins and easily extracted with ether, but at 37°C. Moreover, some radioactivity disappeared from the supernate to the L cell debris. Thus the disintegration of virus particles after contact with L cell was suggested.

Acknowledgement

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A Variant Sendai Virus, Infectious to Egg Embryos but not to L Cells*

Third Report. Growth Characteristics of Myxoviruses in Tissue Culture

By

Nakao Ishida and **Morio Homma**

Department of Bacteriology, School of Medicine, Tohoku University, Sendai ; Prof. N. Ishida

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The system Sendai virus (Myxovirus para-influenzae 1) multiplying in Earle's L cells has been studied first in order to examine the tissue culture cell range of the virus. However, the fact that the virus produced in this system is infectious when inoculated into eggs, has been noticed first. This finding was thought to be rather peculiar in the light of several observations[1)2)] when egg-adapted myxoviruses were tested in various established tissue culture lines, where the results indicated a production of hemagglutinating but not egg-infectious particles.

Thus the growth characteristics of this virus in L cells have been pursued further, in both biological and chemical sense. The present paper, however, concerns on biological findings, with particular emphasis on the characteristics of virus particles produced in this system, which is egg-infectious but not infectious to L cells.

MATERIALS AND METHODS

Virus The Fushimi strain of Sendai virus[3)] ($M_{12}R_1M_3E_{20}$-E_{29}) propagated in the allantoic cavity of 10-day-old chick embryos was used as the inoculum throughout the experiment. To obtain the inoculum, standard passage with 0.2 ml of 10^{-6} diluted fluid was made and incubated at 36°C for 48 hours. This harvest contained $10^{9.5}$ 50 per cent egg infectious doses (EID_{50}) and $10^{3.5}$ hemagglutinins (HA) per ml.

Preparation of L cell cultures Original strain of L cells was furnished by Dr. Katsuta, Institute for Infectious Diseases of Tokyo University and maintained for these 3 years in this laboratory. Growing medium for L cell was con-

石田名香雄, 本間守男

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sisted of 10% bovine serum and 90 % YLE solution. The latter contained 0.5% lactalbumin hydrolysate (Difco) and 0.1% yeast extract (Difco) in Earle's solution with 0.45% glucose. For virus studies, 95% YLE with 5% inactivated horse serum was used as the maintenance solution. This solution did not exhibit any inhibitory action against Sendai virus growth, at least when tested in Maitland's type tissue culture[4] using a piece of chorioallantoic membranes as host cells. This type of tissue culture has been believed to be most sensitive in detecting any growth inhibitors[5]. For propagation of the cell, mature bottle cultures were digested with 0.1% trypsin (NBC, 1 : 300) in Hanks' solution, washed 2 times by Hanks' solution and resuspended in the growing medium. Cell suspension, which concentration is ranging between 150,000 and 200,000 per ml was seeded 1 ml for test tubes (15×160 mm) and 10 ml for standard tissue culture bottles (200 ml), respectively. Cultures were incubated at 36°C, refed with the same growing medium on the fourth day of incubation and used for the study on the 7th day. Satisfactory sheets were constantly formed on that day. Moreover, these cells did not exhibit any altered susceptibility to Sendai virus, throughout the experiment.

Virus inoculation into L cell cultures (Standard technic) To do this, growing medium was drained first from 7–day-old tube or bottle cultures. Then they were washed 2 times with Hanks' solution to remove traces of growing medium. 10^{-1} dilution of the allantoic virus was made in maintenance solution and 1 ml was used for tube culture inoculations and 10 ml for bottle cultures. Each of infected cultures was incubated at 36°C for an hour, then the inoculum was removed as completely as possible, through repeated washings with Hanks' solution. They were refed with fresh maintenance solution again and the virus growth was examined both with the infected cell and with the liquid medium.

Separation of the cell from liquid phase for biological titrations

To test the growth of virus or accumulation of antigens, usually 5 bottles were taken out at determined intervals. Firstly, the supernatant was drained and examined as supernatant fraction after centrifugation at 3,000 rpm for 5 minutes. The cell layer was washed three times with 0.01 M phosphate buffered saline solution of pH 7.2 (PBS) and suspended in PBS with the aid of rubber policeman. These cells were precipitated again by light centrifugation and triturated by means of Potter-Elvehjem's homogenizer in an ice water bath. This homogenate was resuspended into PBS at 5 times concentration of original cultures. The activity was measured with this solution, after light centrifugation. Thus the titers of virus or antigens both in cellular and liquid phase will be described as unit per ml of sample.

Egg infectivity titrations Tenfold serial dilutions of each specimen were prepared in broth and four eggs were inoculated with 0.2 ml of each dilution. After 72 hours of incubation at 36°C, allantoic fluid was removed from each egg and

tested for hemagglutinin. The 50% egg infectious titer (EID_{50}) was calculated by the method of Reed & Muench.

Hemagglutinin titrations In examining the accumulation of hemagglutinins, the tests were performed according to the usual pattern method. Thus, half a ml of diluted material was tested with half a ml of 0.5% chicken red blood cells, at 4°C. However, in analyzing the adsorption rate of the virus to the cell, more critical method proposed by Horsfall and Tamm[6] was followed.

Complement fixation test

Accumulation of soluble antigens inside the cell and also the liberation from the cell were tested by means of complement fixation test. Antibodies were prepared in guinea pigs by instillation of 0.5 ml amount of infected allantoic fluid. Ten days after initial antigen stimulus, S antigen prepared from infected chorio-allantoic membrane was boostered intraperitoneally. The serum was drawn ten days after the last injection and used as V (viral) S (soluble) antibodies. In an effort to detect only S antigens of the tested material, hemagglutinins were adsorbed with packed chicken red blood cell. Thus the preparation without any hemagglutinin activity was examined with VS antibody and the obtained result was understood to be as S antigen titer. The technics to perform the test have been already fully described[7][8].

Hemadsorption test

Hemadsorption technic originally introduced by Vogel and Shelokov for influenza virus[9] was applied in this experiment for the detection of preemergence and/or surface-bound hemagglutinins. After one hour period of incubation under the standard conditions as described above, infected cultures were washed 3 times by Hanks' solution and refed with maintenance solution containing 1% of anti-Sendai virus hyperimmune rooster serum (HAI titer; 1 : 10,240). Further incubation for one hour was made and then, the cultures were washed and refed with fresh maintenance solution. At one hour intervals they were tested for hemadsorption with 0.5% chicken red cell suspensions. Washing the system with immune serum eliminated the misleads due to residual inoculum.

Test for hemolysis

General procedures proposed by Sato[10] were followed throughout the work. In obtaining the starting material, L cell culture fluid infected with Sendai virus was first centrifuged for 30 minutes at 100,000 g. The sediment was resuspended with an aliquot of PBS and the hemolytic activity of this preparation was compared with the counterpart obtained from allantoic fluid. One ml amount of each virus preparation was incubated with an equal amount of washed 2% guinea pig red cell suspension at 4°C. Then the mixture was centrifuged at 3,000 rpm for 2 minutes in refrigerated condition. To this sediment, 2 ml amount of PBS was added and the mixture was incubated at 37°C up to 3

hours. At appropriate intervals, both hemagglutinin titer and grade of hemolysis of the supernatant were pursued.

EXPERIMENTAL

Adsorption rate of the inoculum virus to the cell

In obtaining the full growth of Sendai virus within the L cell, it was first thought advisable to determine the contact period of the inoculum to L cells. Thus the adsorption rate was examined with a definite amount of inoculum virus. For this purpose, a lot of parallel culture tubes were infected with 10^{-2} diluted allantoic fluids, and incubated at 36°C. Under this condition of infection, calculated infectious particles were $10^{7.3}$ in total one ml medium and the average number of cells in one tube was $10^{6.3}$. Three tubes were taken out from water bath at 15, 30, 60, 120, and 150 minutes, respectively and then the culture fluids were removed. Then the cells were washed three times, refed with fresh maintenance solution and incubated for further 96 hours. At the end of this incubation, supernatants of every 3 tubes were pooled and tested for hemagglutinins by the method of Horsfall and Tamm[6]. Thus the time required for full adsorption of Sendai virus to the monolayered culture of L cell was determined not by testing the fraction of inoculum left unadsorbed in the supernatant, but by estimating the number of infectious centers.

TABLE I. Effect of Various Time of Exposure of the Inoculum to the Cell on Virus Growth

Time of exposure (min)	HA titer after 96 hrs. incubation	Virus growth in percentage
15	59	50
30	96	82
60	128	108
120	117	100
150	117	100
Control*	117	100

* Inoculum virus was left all the way through without any washing. Final growth in hemagglutinins was taken as 100%.

Table I shows the results and on the basis of this experiment the time required for 50% adsorption was calculated as around 15 minutes. After 60 minutes, adsorption reached to the maximum in a practical sense. These figures were in good agreement with the value obtained by one of the authors[11] using the PR8 virus and chorioallantoic membrane. On the basis of this experiment, the period of 60 minutes was used for the adsorption of inoculum virus throughout the experiment.

The effect of inoculum size on virus production

In the first place of this experiment, the period necessary for establishing

the infection was determined but with a certain dilution of the inoculum virus. In this place, the effect of different inoculum size on both recovery of virus and the appearance of cytopathic effect were determined by definite period of contact. In performing the experiment, 6 different inoculum size ranging from $10^{8.9}$ to $10^{3.9}$ EID_{50}/ml was prepared in maintenance solution and 3 tubes were infected with each. After the incubation period of one hour, these tubes were washed to remove a trace of the inoculum and refed with fresh maintenance solution. Hemagglutinin production was examined after further 96 hours of incubation. Table II illustrates one of the typical results of the same kinds of experiments,

TABLE II. Different Degree of Cytopathogenicity and Virus Growth with Different Inoculum Size

Inoculum size (EID_{50}/ml)	Score of cytopathogenicity*	HA titer produced after 96 hrs.
$10^{8.9}$	4	1024
$10^{7.9}$	4	512
$10^{6.9}$	4	256
$10^{5.9}$	2	64
$10^{4.9}$	1	3
$10^{3.9}$	0	2

* Degenerative changes of the cell were scored. 75–100% degeneration was taken as 4, 50–75% as 3, 25–50% as 2 and less than 25% as 1.

where reasonable response was found with different inoculum size. Here $10^{6.3}$ cells in average were calculated in one tube. Tubes where $10^{6.9}$ EID_{50} particles were inoculated revealed full marks in degeneration of the cell. With one more diluted inoculum, such a full degenerative change did not follow. Moreover, when we compare the amount of hemagglutinin productions with higher concentration of the seed virus, the recovery of hemagglutinins responsed quite well to the inoculum size. These observations may suggest the one step growth character of the virus in this cell culture but the possibility will be further discussed later. The characteristics of hemagglutinating particles here found will be also described later related to their infectivity, but as far as the experiment here made is concerned, the EID_{50}/HA (I/A) ratio was around 5. Particularly high ratio was not obtainable with diluted inoculum.

In the Table, it is also seen that the seeds could be diluted at most to 10^{-5} in order to produce some evidence of cellular destruction. Thus the virus amount in the order of 10^5 EID_{50} was required to yield some visible cytopathic effect.

Cytopathic effect of the virus

In the above experiment, degenerative changes of the infected cell were scored just by inspecting each tube under the low magnification of microscope, without any staining. To describe the characteristics of this virus-cell in-

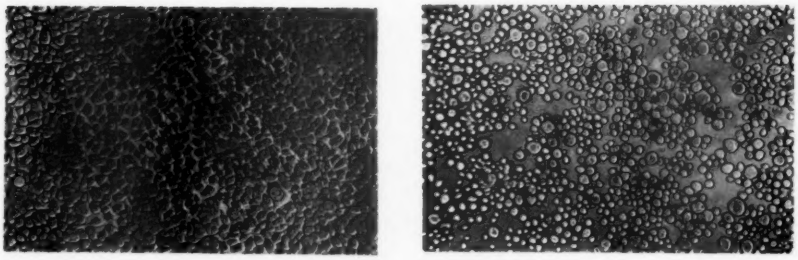

Fig. 1.

Left : Normal L cell. Unstained (×80).
Right : L cell infected with Sendai virus. All of the cells are rounded and some of them still preserve protrusions. 24 hrs. after infection. Unstained (×80).

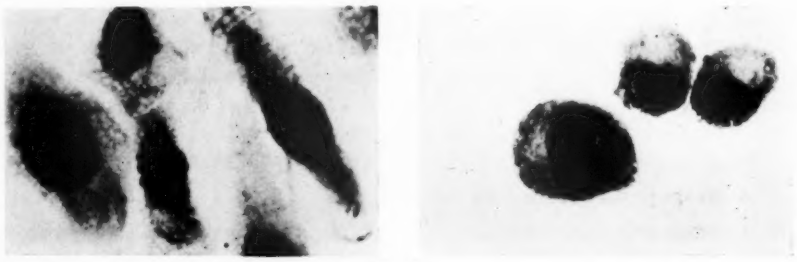

Fig. 2.

Left : Normal L cell. Giemsa stain (×400).
Right : Cytopathic effect of Sendai virus upon L cell.
The cells are rounded and the nuclei are driven to the one side of the cell. Faintly stained area in the juxtanuclear region is readily seen. 24 hrs. after infection. Giemsa stain (×400).

teractions, however, more detailed cytological studies were thought to be a matter of interest. To do this, cover slips were put into each bottle before growing the cell. The rest procedures were made as usual. After infection, these slips were taken out at appropriate intervals and used for staining with Giemsa solution.

Sequential changes of these cells were thus followed under the standard infection condition. Low magnification pictures were illustrated by Fig. 1 and that of high magnification, by Fig. 2. First detectable changes were noticed at 10 or 12 hours after infection. All of the cell showed tendency to rounding in a similar fashion. This kind of effect was never observed at 8 hours. Though they are rounded at 12 or 14 hours, protrusions were still left on both poles (See Fig. 2). No detectable changes were noticed inside the nucleus, but these nuclei revealed the tendency to be driven to one side of the cell. After 18 hours, the protrusions

left at 14 hours were disappeared, thus all the cells were now quite round. Basophilic margination of cytoplasm became gradually apparent from 14 hours up to 18 hours and then the nuclei were driven to the one side of the cell with all of them. After 24 hours, basophilic margination became more prominent and the cells began to slough off from the glass wall. In this condition of infection, almost all of the cells were sloughed off at 96 hours.

The observation described above was obtained at standard inoculation condition. However, when a small inoculum of $10^{5.0}$ EID_{50} virus particles was adopted against $10^{6.3}$ cells, the situation was somewhat different. Infected cells were differentiated from normal cells as a degenerated foci at least from 24 up to 48 hours. However, as time goes, normal cells replaced the parts where infected cells were degenerated. Thus 6 days after infection, these tubes showed appearance which was hardly differentiated from normal one. Nevertheless, when these cells were examined in higher magnification after Giemsa staining, a part of degenerative cells were still preserved as island in the background of normal cell layers. When these cell layers were infected with larger inoculum of virus again, both cytopathic changes and virus multiplication occurred as well as when normal cells were infected. In other words, the cells left on the glass wall was not a refractory fraction of the cell population.

These observations may indicate that the viral multiplication is restricted to the cell originally infected by the seed virus.

Appearance of soluble antigens, hemagglutinins and infectious particles inside the cell

Current thought holds true that in any tissue infected with any myxovirus, the appearance of these three activities may follow in the above order. To test this possibility in the system, culture bottles were infected simultaneously under the standard condition. In each bottle, $10^{8.3}$ infectious particles were inoculated with 10 ml medium, where the number of monolayered cell were $10^{7.3}$ in average. By Fig. 3, one of the typical results obtained from three experiments was illustrated. These repeated experiments, though not always made in an identical fashion, gave us reproducible results.

The first detectable changes within the cell was the appearance of CFS antigen. It was detectable at 6 hours, although the titer was relatively low. Three hours later, measurable hemagglutinin appeared inside the cell and since then the logarithmic increase in titer continued up to 14 hours. At 8 or 10 hours after infection, morphological change of the cell was hardly detectable even when examined after staining. First rise in infectivity titer was demonstrable at 10 hours. However, usual level of residual virus of $10^{3.7}$ has to be taken into account in discussing the first rise. Since then, almost logarithmic increase continued up to 20 hours. With hemagglutinins, however, the logarithmic growth curve was

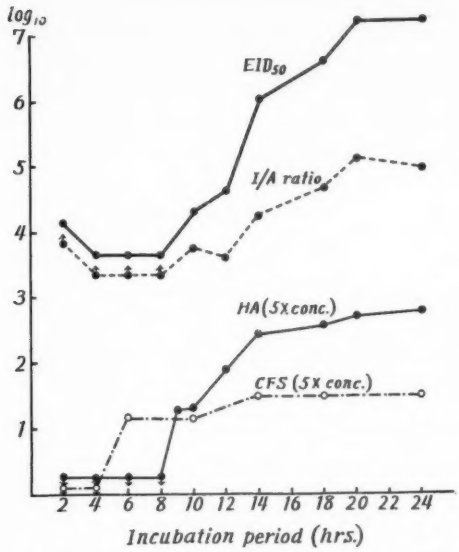

Fig. 3. Antigen Accumulation in L Cells at Earlier Stage of the Infection.

apparent up to 14 hours, then reached to plateau. Thus these two activities did not exactly follow between 14 to 20 hours, and the explanation of this fact will appear later. The fact described above will be easily understood when the I/A ratio was plotted against the time (see Fig. 3). Shift of this ratio was evident from 12 up to 20 hours. The ratio reached to the maximum at 20 hours, where the value still remained around 5.3. With the inoculum virus, propagated in chick embryos, this value was calculated usually as 6.0 or 6.3. Thus, the ratio 3.6 obtained at 12 hours will be easily understood, if one assume the early production of non-infectious hemagglutinins. The existence of this kind of hemagglutinating particle was already noticed when the virus was first isolated[3]. CFS antigen titer reached maximum at 14 hours. But the highest titer was 1 : 6.

Positive hemadsorption found at 7 hours

It was thought as a matter of interest to study the relationship between the time when cyto-hemadsorption and hemagglutinin appear inside the cell.

Up to 6 hours after infection, hemadsorption was hardly observed. However, at 7 hours i.e. 2 hours ahead the detection of hemagglutinin inside the cell, almost all of the cell adsorbed red blood cells uniformly on the surface. From this result, in detecting preemergent or surface-bound virus, hemadsorption technic was believed to be more sensitive than usual test made with tissue homogenate.

Release of hemagglutinin and infectious particles from the cell

In the experiment described above, the accumulation of CFS antigen, hemagglutinin and infectious particles within the cell was examined. From 6 to 10 hours after infection, they were first detectable in the above order. Then the release of these particles into liquid phase was examined but from 0 hour up to 96 hours. The results were shown in Fig. 4. As far as CF antigens were concerned, no

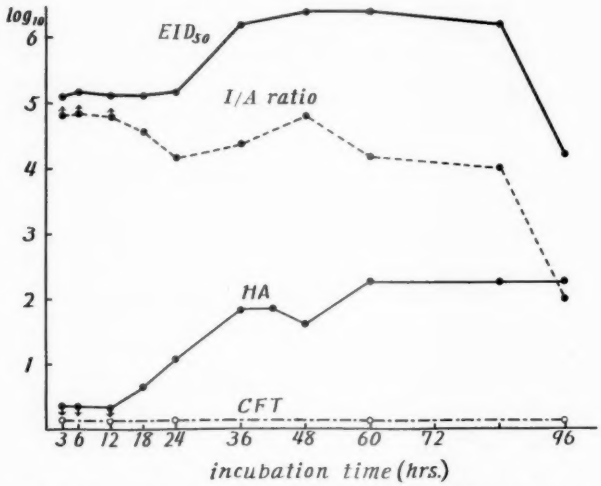

Fig. 4. Virus Liberation from Infected L Cells into the Medium.

titer was detectable in a medium. HA release was detectable from 18 hours and continued up to 36 hours, in a logarithmic fashion. At 60 hours after infection, the production reached to the maximum. Release of infectious particles, however, was first detectable as late as 24 to 36 hours and the titer reached to the maximum at 48 hours. When I/A ratio was calculated again with these liquid samples, 48 hours sample represented highest ratio. Since then, the inactivation of infectious activities was followed.

In comparing the results illustrated by Figs. 3 and 4, one can notice the fact that the release of both HA and EID_{50} into a medium is correlated to the accumulation of both activities inside the cell. For example, logarithmic increase of HA inside the cell ceased at about 14 hours. Then the release of HA into a liquid phase was apparent at 18 hours. Thus the liberation of HA was first detectable at 18 hours, where the HA production in the cell reached to plateau. Infectious particles inside the cell reached to the maximum at 20 hours and the release was evident from 24 to 36 hours. To understand the events occuring both inside and outside the cell, Fig. 5 was drawn. In the figure, morphological changes of the cell

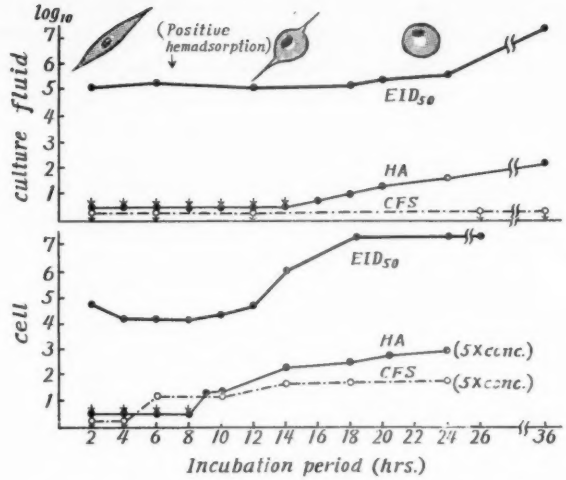

Fig. 5. Virus Multiplication in Cellular and Liquid Phase and Development of Cytopathogenicity.

were also involved. Up to 8 hours, the cells preserved their normal appearance. However, the positive hemadsorption occurred at 7 hours. If one wishes to correlate the morphological changes to the events occuring in the cell, the morphological changes started when the accumulation of infectious particles was first detectable. When these titers reached to the maximum, all cells revealed chracteristic basophilic margination of the cytoplasm. Since then, the cell on the glass wall began to slough off at 24 hours of incubation and then the titer of egg infectious activities increased in the medium.

Failed attempts to propagate the L cell-grown virus again in L cells

Virus particles which have been accumulated inside the L cell or released from the cell, were apparently infectious as far as chorioallantoic cavity of embryonated egg was used for their reproduction. However, their infectiousness to the L cell was thought to be rather doubtful. Because, in the second experiment described above, where the virus growth and the appearance of cytopathic effect in response to the infection with different inoculum size were examined, both activities corresponded rather exactly to the amount of inoculum virus. If the second cyclic growth were occurred, this kind of effect will not be expected. Moreover, the fact was again confirmed when the cytological changes were pursued in detail, where the foci once appeared after the infection with the low inoculum did not develop in diameter nor increase in numbers. Rather the healthy cell population adjacent to the foci overgrew these areas. Having

this kind of consideration in mind, possibility of the Sendai virus transmission from L cell culture to L cell culture was examined in a more elaborated manner. Harvests obtained at 48 hours of first L cell culture, where the I/A ratio was highest among these liquid specimens were concentrated by centrifugation at 40,000 g. These materials of high egg infectious titer were tested for their infectivity again in new L cell cultures. Production of the virus in the second L cell culture was pursued by means of both HA and egg infectivity. Detailed conditions to do this and the results were shown in Table III. From the Table, it is evident that the

TABLE III. Secondary Passages of L Cell-grown Virus

Time of incubation (hours)	EID_{50}	HA titer
Inoculum*	$10^{8.0}$	$2^{10.5}$
8	$10^{5.5}$	$<2^{1.0}$
72	n. d.†	$<2^{1.0}$
96	$<10^{1.2}$	$<2^{1.0}$

* Concentrated 10 times by ultracentrifugation
† Not done

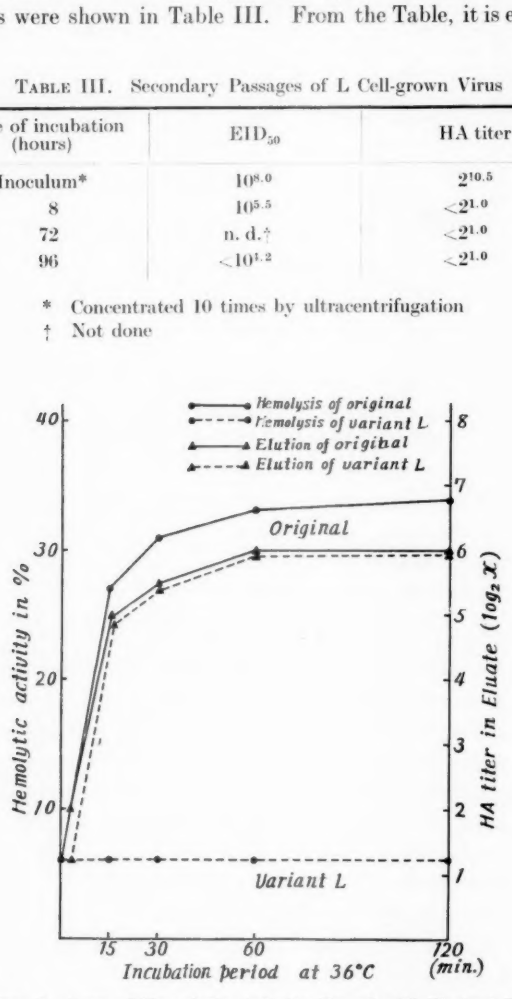

Fig. 6. Loss of Hemolytic Activity Found with Variant L.

growth in the second L cell culture is failed. Addition of allantoic fluid to the L cell-grown virus did not increase its infectivity, so that the difference is probably not due to traces of some non-specific factor from the eggs.

Loss of hemolytic activity with virus propagated in L cells

Decrease in I/A ratio of the virus population produced in L cells have been described above. Ultracentrifugal studies to be described elsewhere[12] on these particles, also revealed that these particles are somewhat lighter than the usual 950 S component of the original virus produced in chick embryos. But the fact will not be a explanation of the non-transmissible character of the virus produced here, in the light of experiment where the virus of low I/A ratio but propagated in eggs is shown to be infectious to the L cell. Then the alternative explanation of the fact will be a kind of host-controlled variation which may occurred in the L cell. To test this kind of possibility, many markers, such as enzymatic activities, hemolytic activities, heat-lability of hemagglutinins, and hemagglutinin spectrum with various red cells have been compared between the virus propagated in chick embryos and in L cell cultures. As a result, only one difference, noticed with L cell-grown virus (Variant L), was the lacking of hemolytic activity. Fig. 6 illustrates the result obtained along this line. The eluting activity of the Variant L was well preserved as the original allantoic fluid virus. However, the helmolytic activity of the former was completely lost.

DISCUSSIONS

Growth characteristics of the Sendai virus among the population of myxoviruses have been understood as the long latent period for its reproduction. However, this fact has been shown both in chick embryos[13] and in tissue cultures[14] using chorioallantoic membrane as the host cell. Recently, Shimizu *et al.*[15] had a chance to test the propagation of various strains of HVJ and their cytopathic effect in primary swine kidney tissue culture. However, they did not refer to this respect. Moreover, descriptions were never made on the growth of this virus in any established tissue culture cell lines.* From this reason, growth of this virus was tested in HeLa, L and FL cells. As a result similar cytopathic effect and growth characteristics have been obtained with this virus in the above 3 cell lines. Moreover, the long latent period, which will be preferable in performing chemical studies were also confirmed in these tissue cultures. The study, however, was made extensively with L cell cultures, from two reasons. Firstly, only this cell is originated from mice among the above three. Extensive studies

* When this manuscript was prepared for application, Tyrrell's paper[21] on the growth of influenza virus, including Sendai, in various kidney cell cultures appeared. Essentialy the same result as reported here was involved, although he used again the primary cell cultures.

on the mode of murine influenza was recently made in this laboratory with PR8 virus[7] and tissue culture studies[16] of various mouse organs were performed to explain the pneumotropism of the myxovirus. In this very sense, L cells were preferable than the remaining two. Secondly, the cytopathic effect occurred in the L cell was favourable than the other two in directing the experiment, where morphological changes of the cell among the population occurred simultaneously and in the uniform fashion.

Characteristics of the virus growth in the system will be summarized into two respects. Firstly, though the complement fixing antigens, hemagglutinins and infectious particles have been produced in this time sequence as were the case with other systems, the time when they were first detectable was between 6 and 10 hours. In other words, dark period required to detect various activities of this virus was longer in general than other known related reactions. However, this fact will be further criticized in the next paper[17], where fluorescent antibody technics were used in revealing these activities. This technic was quite sensitive and at least at present, we know the fact that accumulation of V antigens in the cytoplasm is detectable as early as four hours after infection[17].

Quite recently, many people are inclined to believe that Sendai virus is related to mumps and Newcastle disease virus (NDV) on the basis of their hemolytic activity and shared antigenicity[18][19]. Growth characteristics of these viruses in tissue cultures are also related but the fact has to be examined further in detail. Reproduction of mumps virus in HeLa cells[20], particularly when they are freshly isolated is already known. Sendai virus has been proved here to produce egg infectious particles in the 3 established cell lines, provided sufficient quantities of virus were added. Cytopathogenicity of Sendai virus could be readily demonstrated and they were differentiated from that of mumps virus or NDV. However, in discussing the sensitivity of different tissues to detect Sendai virus, chick embryos are believed to be more favourable.

Loss of infectivity to the L cells and loss of hemolytic activity noticed with particles released from L cell will be the most interesting matter to be discussed. The fact was not explainable by the selection of mutant, because the particles lacking in infectivity to L cells and hemolytic activity were produced only one passage of egg adapted virus in L cells and when these viruses produced in L cells were diluted in a manner to test the single fluctuation and when they were propagated in chick embryos again, they acquired these two activities. Thus the nature of host-controlled variation will be plausible as an explanation, at least for a time being. Recent observation made in this laboratory on the latent infection of hemadsorption type 2 virus in HeLa cell cultures, also revealed the same fact(unpublished).

SUMMARY

Growth characteristics of the Sendai virus in Earle's L cell have been studied. As long as 6 hours was necessary to detect soluble antigen in the cell. Positive hemadsorption was found at 7 hours. Accumulation of soluble antigen, hemagglutinin and infectious particles were detectable in this time sequence but almost from 6 to 10 hours. Non-infectious hemagglutinins were first detectable inside the cell and the release of these kinds of particles were ahead to the liberation of infectious particles. As a result, obtained harvest had the low I/A ratio, when compared to the virus population obtained after reproduction in chorioallantoic cavities of chick embryos. A kind of host-controlled variation has been observed with the particles liberated from L cells. They were infectious when inoculated into eggs but not infectious when inoculated to L cells again. They also lost the hemolytic activity which had been believed to be a dominant character of this virus, at least when grown in chick embryos.

Acknowledgement

The authors wish to express their heartful thanks to Dr. Kuroya, emeritus professor of this school, for his encouragement throughout the work.

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Effect of Acute Hypothermia upon the Noradrenaline and Adrenaline Content of the Adrenal Gland in the Cat

By

Mitsuki Ide

From the Department of Physiology, Nagasaki University School of Medicine, Nagasaki

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In my previous studies the effects of insulin hypoglycemia[1] and hemorrhagic hypotension[2] upon the noradrenaline and adrenaline contents of the cat adrenal gland were investigated. The purpose of the present study has been to know the influence of acute hypothermia induced by the application of cold to the animal body upon the adrenal noradrenaline and adrenaline contents.

EXPERIMENTAL

Methods and Results

Two groups of 4 male and 4 female cats each were used. All the animals were anesthetized with Evipan sodium.

One group of cats were immersed into cold water of 6–8°C for 30 minutes and then withdrawn from it. One and a half hours after withdrawal from cold water the adrenal glands of both sides were extirpated. The other group of cats were used for the control experiments, the adrenal glands being removed 2 hours after Evipan sodium injection.

Adrenal extracts were made with 4% trichloracetic acid and were assayed for noradrenaline and adrenaline by the permanganate method of Suzuki and Ozaki[3].

All the data are presented in Table I.

In control experiments the adrenal adrenaline and noradrenaline contents were on the average 0.63 μg. and 0.61 μg. per mg. of gland weight, respectively. They were 44 μg. and 42 μg. per kg. of body weight, respectively. The mean relative noradrenaline content was 50 per cent.

In cats, immersed into cold water, the body temperature fell from 37.4–38.5°C markedly. Ten or 20 minutes after withdrawal from cold water the lowest level (24.2–27.1°C) was reached. Then it rose very slowly. One and a half hours after withdrawal from cold water it was measured as 26.7–31.3°C. In

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TABLE I. Effect of Cold Application to the Cat Body on the Noradrenaline and Adrenaline Contents of the Adrenal Gland

No.	Body weight (kg.)	Sex	L or R	Weight of gland (mg.)	Adrenaline and noradrenaline content (μg.)						Per cent noradrenaline
					per gland		per mg. of gland		per kg. of body weight		
					Adr.	Noradr.	Adr.	Noradr.	Adr.	Noradr.	
Control experiments											
1	2.35	♂	l	134	106	102	0.79	0.76	45	43	49
			r	136	109	102	0.80	0.75	46	43	48
2	2.06	♂	l	209	132	90	0.62	0.43	64	44	41
			r	190	130	91	0.68	0.48	63	44	41
3	2.48	♂	l	268	104	94	0.39	0.25	42	38	47
			r	282	106	97	0.37	0.34	43	39	48
4	2.18	♂	l	192	101	124	0.53	0.65	46	57	55
			r	193	96	115	0.50	0.60	44	53	55
5	1.68	♀	l	84	82	57	0.98	0.68	49	34	41
			r	76	75	54	0.99	0.71	45	32	42
6	4.10	♀	l	211	84	140	0.40	0.66	20	34	63
			r	205	90	122	0.44	0.60	22	30	58
7	3.21	♀	l	251	130	142	0.52	0.57	40	44	52
			r	228	119	141	0.52	0.62	37	44	54
8	2.44	♀	l	140	124	134	0.89	0.96	51	55	52
			r	149	103	106	0.69	0.71	42	43	51
			Mean		**106**	**107**	**0.63**	**0.61**	**44**	**42**	**50**
Cold application experiments											
9	2.23	♂	l	290	118	108	0.41	0.37	53	48	48
			r	281	108	132	0.38	0.47	48	59	55
10	2.42	♂	l	126	62*	83	0.49	0.66	26	34	57
			r	123	55	82	0.45	0.67	23	34	60
11	2.25	♂	l	111	49	63	0.44	0.57	22	28	56
			r	105	31	45	0.30	0.43	14	20	59
12	2.12	♂	l	192	79	98	0.41	0.51	37	46	55
			r	199	89	94	0.45	0.47	42	44	51
13	2.27	♀	l	154	47	54	0.31	0.35	21	24	53
			r	151	38	55	0.25	0.36	17	24	59
14	3.83	♀	l	164	31	92	0.19	0.56	8	24	75
			r	171	27	105	0.16	0.61	7	27	80
15	2.83	♀	l	198	99	121	0.50	0.61	35	43	55
			r	195	88	110	0.45	0.56	31	39	56
16	2.24	♀	l	120	80	95	0.67	0.79	36	42	54
			r	114	65	79	0.57	0.69	29	35	55
			Mean		**67**	**89**	**0.40**	**0.54**	**28**	**36**	**58**

this group of cats the adrenaline and noradrenaline contents of the adrenal gland were on the average 0.40 μg. and 0.54 μg. per mg. of gland weight, respectively. They were 28 μg. and 36 μg. per kg. of body weight, respectively. The mean relative noradrenaline content was 58 per cent.

The adrenaline content of the adrenal gland in the hypothermic cats was

definitely smaller than that in control animals. The difference was statistically significant ($P<0.01$). The noradrenaline content of the adrenal gland in cats immersed into cold water seemed to be somewhat smaller than that in control animals. This difference was, however, statistically not significant ($P>0.05$).

DISCUSSION

It was well established that adrenal medullary hormone content of the adrenal gland was decreased by cooling the animal.[4-6] However, no exploration has been performed to evaluate differentially the effect of cold upon the adrenal noradrenaline and adrenaline contents.

In the present study it has been elucidated that by the application of cold to the animal body the adrenaline content of the adrenal gland is decreased, whereas there is no definite reduction in the adrenal noradrenaline content.

In the previous study[1] it was found that the adrenaline content of the adrenal gland in the cat was reduced definitely by the administration of insulin in a dose of 2 units per kg. of body weight, while no definite change in adrenal noradrenaline content was produced. After giving insulin in a dose of 5 units per kg. of body weight, both adrenaline and noradrenaline contents of the adrenal gland decreased definitely, the adrenaline depletion being much more remarkable than that of noradrenaline.

Similar results were obtained in the hemorrhage experiments[2] in cats. After bleeding two-fifths of the total blood quantity, a definite decrease in adrenaline content of the adrenal gland was observed. However, no decrease in the adrenal noradrenaline content was found. In cats, in which blood was taken out at first in an amount of two-fifths of the total and after one hour again in a volume of one-fifth of the total, there was a marked decrease in adrenaline content and a moderate reduction in noradrenaline content of the adrenal gland.

The above experiments and the present study showed that insulin hypoglycemia, hemorrhagic hypotension and cold stress reduced adrenaline content of the adrenal gland rather than noradrenaline content.

SUMMARY

The noradrenaline and adrenaline contents of the adrenal gland in cats were estimated by the permanganate method of Suzuki and Ozaki. Hypothermia was induced by immersing the animals into cold water.

After application of cold to the animal body a marked decrease in adrenaline content of the adrenal gland was found, whereas there was no definite alteration in adrenal noradrenaline content.

The author wishes to express appreciation to Prof. T. Suzuki for his interest and to Dr. M. Motomura for his help in conducting experiments.

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The Conduction Velocity of the Ulnar Nerve and the Spinal Reflex Time Measured by Means of the H Wave in Average Adults and Athletes

By

Masamichi Kato

Department of Physiology, Hokkaido University, School of Medicine, Sapporo, Japan

(Received for publication, June 20, 1960)

Numerous papers on the conduction velocity of the nerve fibers have been published since the original report of Helmholtz and Baxt (1883). Among these papers, an experimental result was presented by Wagman and Lesse (1952) that the maximum conduction velocity of human ulnar nerve fibers may be attained as early as the fourth to fifth years of life and that it may not increase from these years onwards with the growth of the body. Their result is in agreement with Rexed's (1944) histological data that the fiber size distribution in the ventral root attains the adult distribution between the ages of five and nine years.

Recently Thomas and Lambert (1960) have measured the conduction velocity of the ulnar nerve as well as the spinal reflex time by means of the H wave on human subjects and have reached the same conclusion as Wagman and Lesse.

In the present experiments, the maximum conduction velocity of ulnar nerve fibers and the spinal reflex time in the tibial nerve by means of the H-wave have been measured, in the first place, on 30 average adults under the special consideration of room temperature. In the second place, they have been measured also on 38 selected athletes for comparison with above noted data in order to elucidate whether the maximum conduction velocity of nerve fibers could increase following physical exercise during youth.

METHODS

Experiments were carried out on 30 average adults, i.e. ordinary university students (20–30 years of age) showing no neuromuscular disorders, and on 32 student athletes of Hokkaido University who have more than 5 years history as athletes and have most skillful techniques in each kind of sport (viz., 7 "Kendo" participants, 5 rugby players, 5 volley-ball players, 5 badminton players, 5 football players and 5 "Karate" participants), 4 semiprofessional "Kendo" (Japanese

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fencing) experts and 2 professional "Sumo" wrestlers who belonged to the "Kokugikan." Their ages ranged from 20 to 36 years.

As to the instrumentation of the experiment, electronic stimulator (Type ES-103, San'ei Instrument Co.), double beam oscilloscope (Type UB-203B, San'ei Instrument Co.) and Canon camera were used for stimulation of the nerve fibers and for recording of the evoked myograms.

In a series of experiments on the conduction velocity of the ulnar nerve, supramaximal square wave stimuli (0.5 msec. duration, 0.5 per second frequency) were applied percutaneously to the ulnar nerve at the axilla, elbow and wrist. Evoked myograms were picked up from the hypothenar eminence muscles. For the stimulation, a silver disc of 10 mm diameter covered with lint with a short ebonite handle was used as a cathode. The electrode soaked with saline was applied over the ulnar nerve with a constant pressure at the above-noted three points. An indifferent electrode was a silver plate of 3×6 cm^2 covered with lint and soaked with saline; it was placed on the dorsal aspect of the upper arm. For picking up evoked myograms, a pair of silver discs of 10 mm diameter were employed. They were attached with electrode paste to the belly of the hypothenar eminence muscles and on the proximal pharanx of the little finger respectively.

Measurement of the latencies of evoked myograms was made from the average

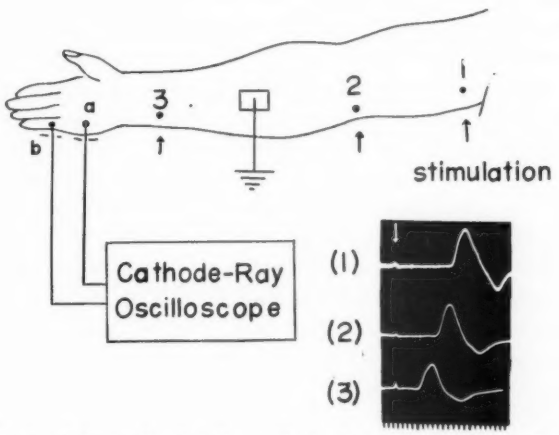

Fig. 1. Schema showing the method of measuring the conduction velocity of the ulnar nerve fibers.

Stimulations are applied to the ulnar nerve trunk at three points, 1, 2 and 3; the axilla, elbow and wrist successively and the action potentials of the hypothenar eminence muscles were picked up. Records (1), (2) and (3) show the evoked electromyograms obtained by stimulating the corresponding number of the points 1, 2 and 3. Time mark : 1 msec.

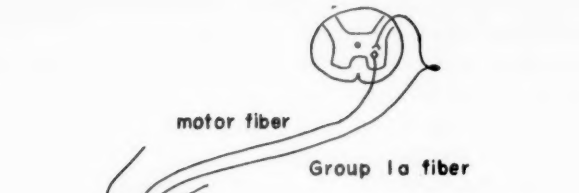

Fig. 2. Schema showing the method of measuring the spinal reflex time.

The M and H waves are obtained by stimulating the posterior tibial nerve at popliteal fossa. Time mark : 10 msec.
Latency of the H wave was measured at its maximum amplitude.

value of three recordings at each stimulating point, and the conduction velocity of the most rapidly conducting ulnar nerve fibers was calculated from the differences of latencies and the distances between the points of stimulation. The data were obtained separately in the forearm and upper arm.

The arrangements for the experiments are shown in Fig. 1.

Another series of experiments on the spinal reflex time were performed on each subject one after another after the above-noted experiments. Square wave stimulation was applied percutaneously to the posterior tibial nerve at the popliteal fossa using the same electrodes and with the same wave form and frequency as in the case of the ulnar nerve described above. The stimulating point was carefully selected where the H wave was most easily observed. The intensity of stimulation was controlled so that the maximum amplitude of the H wave could be obtained. The H and M waves were picked up from the middle of the belly of the lateral gastrocnemius muscle by means of monopolar lead, the indifferent electrode being placed on the instep. The electrodes used were the same as in the previous series of experiments. The subjects lay prone on the bed with the legs relaxed and ankle joint resting on a pillow at an angle of about 120°.

The experimental set-up is shown in Fig. 2.

Throughout the above-noted two series of experiments, the room temperature was carefully controlled at about 30°C (27°C–35°C) by stove.

Skin temperature was measured at several points in the upper and lower extremities along their length by thermister devices directly after the experiments.

RESULTS

1. Effect of temperature upon the conduction velocity of the ulnar nerve

As in a preliminary experiment, the effects of environmental temperature upon the conduction velocity of the ulnar nerve were investigated on 5 subjects. Environmental temperature was changed from 10°C to 45°C in four stages successively by means of the following procedures: Firstly, forearm covered with vinyl cloth was kept in cold water of about 10°C for 15 minutes, secondly it was exposed to the room temperature of about 20°C for 20 minutes, thirdly the room temperature was gradually raised to about 30°C by stove and at last the forearm was kept in a box warmed by an electric lamp at about 40°C for about 15 minutes. The measurement of conduction velocity was made at the end of each stage of controlled environmental temperature when the skin temperature was proved to have reached its equilibrium.

As a result, the conduction velocity increased significantly as the environ-

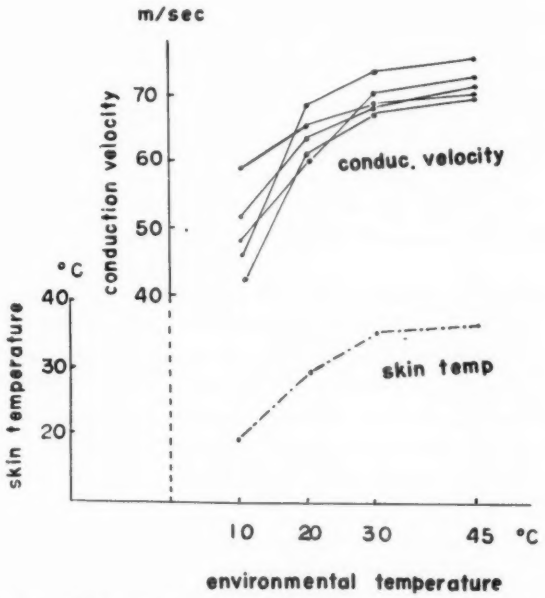

Fig. 3. Effect of environmental temperature on the conduction velocity of the ulnar nerve.

Conduction velocity increased in each case with the rise of environmental temperature. The grade of rise is steep at lower temperatures. The skin temperature at wrist rose likewise.

mental temperature was raised from 10°C to 30°C ,whereas no significant difference was observed between the data of 30°C and 45°C, as shown in Fig. 3. The mean conduction velocities were 49.0±8.2 m/sec., 63.8±4.5 m/sec., 69.1±3.1 m/sec. and 71.1±3.2 m/sec. at 10°C, 20°C, 30°C and 45°C respectively (± indicates the confidence limit at P=0.05).

As to the skin temperature, its distal lowering was observed at the room temperature of 20°C, i.e., the mean value at the wrist (24.0°C) was lower than that at the elbow (27.4°C), while no regional difference was recognized at 30°C.

Another preliminary experiment on 3 subjects has revealed that the variation of the conduction velocity measured on 4 different days was negligible on each subject when they were measured at the room tempearture of 30°C, viz., their differences were within 6% of the velocity.

On the basis of these experimental results and of Aschoff's data (1958) that temperature gradient from core to skin is small at the room temperature around 30°C, experiments were carried out thereafter at the room temperature of about 30°C.

2. The conduction velocity of the ulnar nerve in ordinary students

The average conduction velocity of the ulnar nerve in 30 ordinary students was 67.0±2.5 m/sec. (55.0–79.3 m/sec. : range of variation) and 67.6±2.6 m/sec. (57.2–78.2 m/sec.) in the forearm and upper arm respectively. It was noticed that no significant difference existed between the data of the forearm and upper arm.

Regional difference of the skin temperature was not very marked as they remained within 1.5°C even between the data at the axilla and the wrist in each subject.

Distinct individual differences in the conduction velocity were observed as judged from the above-noted range of variation in the parentheses. Therefore an attempt was made to investigate their cause by examining the correlation between the conduction velocity and various factors such as body height, body weight, thickness of the upper and lower extremities, grasping power and power of back muscles. All these examinations showed that such factors had no effect.

3. The spinal reflex time in ordinary students

Two kinds of evoked myograms, the H and M waves of Magladery and McDougal (1950), are obtained from the gastrocnemius and soleus muscles by stimulating the posterior tibial nerve at popliteal fossa. The H wave with longer latency is elicited by the impulses which are produced in the sensory nerve (group Ia fibers) at the stimulating point and travel through motor fibers to the muscle over the spinal reflex arc, whereas the M wave with shorter latency is due to the direct impulses of the motor fibers from the stimulating point.

These two waves have the following characteristics as was noted by Magladery and McDougal (1950); as the intensity of the stimulation to the tibial nerve is progressively increased, the H wave appears first and increases in amplitude to a certain extent and then dwindles gradually until at last it disappears, whereas the M wave increases in amplitude consistently until the maximum amplitude is reached.

In the measurement of the spinal reflex time, the latency of the maximum H wave was measured as the spinal reflex time in this experiment. The mean spinal reflex time of 30 ordinary students was 26.2±0.4 msec. (22.0–29.5 msec.). A high correlation was recognized between the spinal reflex time and the distance of the reflex arc (correlation coefficient; +0.71), the latter being measured as the distance between the stimulating point at the popliteal fossa and the spinous process of the fourth lumbar vertebra plus the distance between the stimulating point and the site of the recording electrode in the belly of the lateral gastrocnemius muscle.

From this result and from the theoretical viewpoint that most of the reflex time may be consumed by the impulse in travelling the nerve fiber, the spinal reflex index, which may correspond to the mean conduction velocity of the above-mentioned reflex arc, was calculated by means of the following equation:

$$\text{Spinal reflex index} = \frac{\text{distance of the spinal reflex arc}}{\text{spinal reflex time}}$$

TABLE 1. Conduction Velocity of the Ulnar Nerve in the Athletes Groups and Ordinary Student Group

	forearm (m/sec.)	upper arm (m/sec.)
"Kendo"	(7) 64.8± 6.0	67.6± 5.5
Rugby	(5) 65.1± 5.2	63.6± 8.9
"Karate"	(5) 68.8± 6.3	68.0±25.9*
Volley-ball	(5) 66.5±12.9	72.2±10.6
Football	(5) 65.2± 9.3	70.2± 7.2
Badminton	(5) 66.0± 9.1	66.0±12.1
"Kendo" experts	(4) 64.9± 6.1	66.1± 8.5
"Sumo" wrestlers	(2) 65.9**	68.6±24.7
Total	(38) 66.5± 2.3	67.2± 2.6
Ordinary students	(30) 67.0± 2.5	67.6± 2.6

± means the confidence limit at the significance level of 0.05.
* includes only three subjects.
** includes only one subject.

No significant differences are seen among individuals of each section of the athletes group.

Any significant differences are observed neither between the data in the forearm and upper arm both in athletes and ordinary student groups, nor between the two groups.

The mean value of this index in the ordinary student group was 45.3±0.6 (41.2–48.2).

4. The conduction velocity of the ulnar nerve in the athletes group

The mean conduction velocities of the ulnar nerve in each group of various kinds of sport are shown in Table 1. Since there were no significant differences among them, the mean value of all the athletes was calculated as 66.5±2.3 m/sec. (51.0–79.0 m/sec.) and 67.2±2.6 m/sec. (52.5–82.0 m/sec.) respectively in the forearm and upper arm. No significant difference between the data of the forearm and upper arm was observed as in the case of the ordinary student group. It was also noticed that no significant difference was observed between the above-noted data in the athletes group and those in the ordinary student group (67.0±2.5 m/sec. and 67.6±2.6 m/sec.).

The distribution of the conduction velocity of both groups is illustrated in

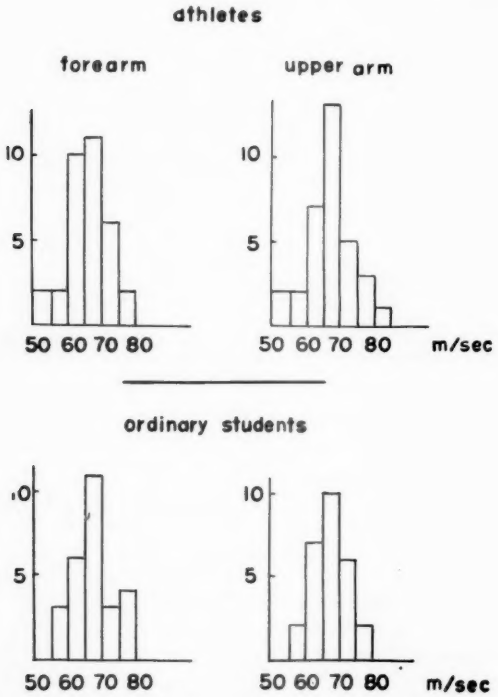

Fig. 4. Distributions of the conduction velocity of the ulnar nerve in athletes group and ordinary student group.

They are all of normal distribution pattern and resemble each other.

Fig. 4. The graphs show almost the same type of normal distribution.

As to the individual differences, distinct ones were observed in the athletes group, too. Therefore, an attempt was made to find out if there is any relationship between the conduction velocity and skillfulness in the sport or the length of history of sports life on individual subjects. For this purpose, subjects of most skillful techniques and of extraordinary long history of sport or inversely subjects of most rapid conduction velocity were selected. These examinations resulted in all negative.

5. The spinal reflex time in the athletes

Among mean values of each group of various kinds of sport, no significant difference was recognized in the spinal reflex time. The mean value for all the athletes was 26.3 ± 0.5 msec. (24.0–29.7 msec.). Close correlation was also recognized between the reflex time and the above-noted distance in the athletes group as is shown in Fig. 5 (correlation coefficient; + 0.77).

It was noticeable that in the spinal reflex time there was no significant dif-

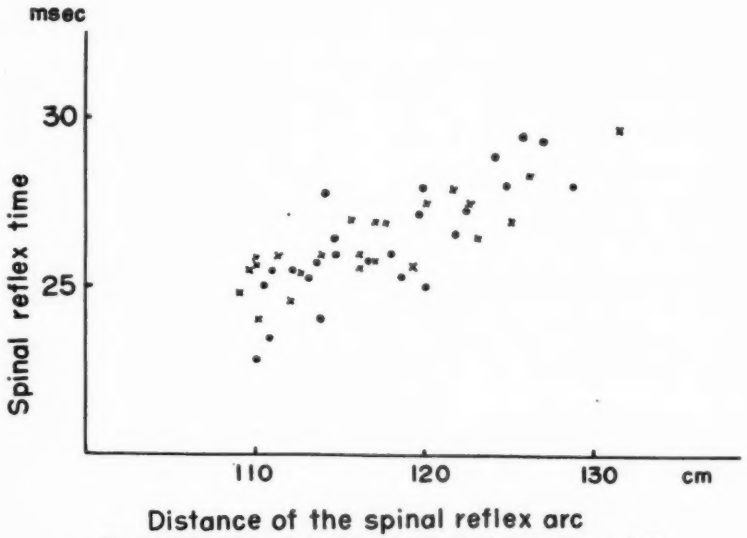

Fig. 5. Correlation chart between the spinal reflex time and the distance of the reflex arc.

● indicates the values of the ordinary student group.
× indicates the values of the athletes group.

High correlations are seen between two data in both of the groups. No significant difference in the spinal reflex time is presumed in two groups, since the distributions of two groups intermingle with each other.

ference between the athletes group and the ordinary student group, as was also the case with the conduction velocity of the ulnar nerve. Fig. 5 also shows evidence that there is no significant difference in the spinal reflex time between the two groups, the patterns of distribution of the spinal reflex time being fairly well overlapped with each other.

DISCUSSION

The effect of room temperature must be considered in measuring the conduction velocity of the peripheral nerve fibers, since it was reported by many authors that the conduction velocity of nerve fibers decreases with lowering of temperature; by Helmholtz and Baxt (1883) in man, by Gasser (1931) in green frog, by Chatfield *et al.* (1948) in golden hamster and in albino rat, and by Tasaka *et al.* (1958) in man.

Distal slowing of the conduction velocity due to distal lowering of the skin temperature at low room temperature may be one of the main troubles in measuring the conduction velocity of peripheral nerve. In this respect, Dawson (1956) stated that on account of the gradient of temperature along the long axis of the limb it is difficult to measure the absolute value of the conduction velocity of nerve fiber in human subjects.

In the present preliminary experiments it was confirmed that the conduction velocity of the ulnar nerve increased with the rise of room temperature, although the ratio of increase became less at about 30°C of room temperature, and that almost the same values were obtained on different days of experiments when they were measured at about 30°C room temperature, no distal lowering of the skin temperature being observed at such a high room temperature as 30°C.

On the basis of these experimental results, room temperature was kept at about 30°C throughout the present experiments.

Carmichael *et al.* (1941) measured the conduction velocity of the sympathetic nerve fibers likewise, i.e., at room temperature of 45°C.

As regards the conduction velocity of the ulnar nerve fibers in the forearm of human subjects obtained by Wagman and Lesse (1952), the mean value of 35 subjects (20–30 years old) was 58.4 ± 4.28 m/sec. (± 4.28 means standard deviation) at an uncontrolled room temperature, by Tasaka *et al.* (1958) the mean value of 54 subjects (aged 22–40 years) was 63.6 ± 6.0 m/sec. at room temperature above 20°C, by Thomas *et al.* (1959) the mean value of 77 subjects (18–65 years old) was 56.1 ± 4.7 m/sec. in the ordinary room temperature and by Thomas and Lambert (1960) the mean value of 188 adults (aged 16–63 years) was 60.0 ± 0.4 m/sec. (± 0.4 means standard error).

It must be noticed that these data are all smaller than those obtained in the present experiments. This may be atrributed to the fact that they were measur-

ed at room temperature around 20°C, that is about 10°C lower than that of the present experiment. This difference in the conduction velocity seems to be within the range conceivable from the present preliminary experimental result, the difference of conduction velocity between temperatures of 20°C and 30°C being 5.3 m/sec.

According to Magladery and McDougal (1950), the conduction velocity of ulnar nerve was by about 10 meters per second slower in the lower forearm than in the upper forearm, and the branching of nerve fibers was suggested to be the cause of the distal slowing. However, no significant difference in the conduction velocity between the upper arm and forearm was observed either in the ordinary student group or athletes group in the present experiments conducted at room temperature of about 30°C where no distal lowering of the skin temperature was recognized. On the other hand, tapering or branching in peripheral nerves as a factor of distal slowing of the conduction velocity has been denied statistically by Rexed (1944) in human subjects, although evidence of tapering of peripheral nerve fibers in frog was presented by Dunn (1902). Furthermore, Eccles and Sherrington (1930) found that branching of motor fibers in cat was greatest at a short distance from the muscle, but not so marked beyond 1 cm from the muscle. The finding of Causey and Schoepfle (1951) obtained on the tibial nerve of rabbit may also support the denial of distal slowing of the conduction velocity of nerve fibers; conduction velocities of the tibial nerve were approximately 56 m/sec. in both proximal and distal stretch.

As regards the spinal reflex time measured by means of the H wave, Magladery *et al.* (1951) measured the latency of the H wave. In the present experiments, latencies of the H wave were measured both in the ordinary student group and in the athletes group with the result that no significant difference was seen between the data derived from the two groups.

Spinal reflex time was calculated in the present experiment for the purpose of obtaining a datum which would correspond to the mean conduction velocity of the impulses over the whole spinal reflex arc.

Another method to measure the spinal reflex time was tried in the present experiment; the difference of latencies of the maximum M and maximum H waves was measured. However, there may exist an uncertainty in this method that the fastest reflex impulse does not necessarily traverse the largest motor fibers, even if latencies of the M and H waves are measured respectively at their maximum amplitudes. At any rate, the spinal reflex time is also a problem of the conduction velocity of the nerve fibers because most of the reflex time may be consumed for the impulses to traverse the sensory and motor fibers, the time for monosynaptic transmission being believed as short as one msec.

As to the individual difference in the conduction velocity of the ulnar nerve in average adults, vairous factors which might cause such difference were in-

vestigated with negative result.

In regards to the effect of physical exercise upon the conduction velocity of nerve, no significant difference in the conduction velocity of the ulnar nerve in the spinal reflex time or in the spinal reflex index was observed between the ordinary students and athletes groups.

Distinct individual differences in the conduction velocity of the ulnar nerve and in the spinal reflex time were observed in the athletes group, too. However, no evidence was found that physical exercise might cause this individual difference.

From these results, it was concluded that the maximum conduction velocity of peripheral nerve fibers might be attained in the childhood as was reported by Wagman and Lesse (1952) and that physical exercise in the youth by sport might not alter the conduction velocity any more. Therefore, rapid action and quick reflexes in athletes may be attributed to the function of the central nervous system.

SUMMARY

An attempt was made to ascertain whether physical exercise might cause increase of the conduction velocity of the nerve fibers in young adults.

Experiments were carried out on 32 university student athletes and 6 professional athletes with controls of 30 ordinary university students. The ages of these subjects ranged from 20 to 36 years.

For the purpose of measuring the conduction velocity of the ulnar nerve, square wave stimulation of 0.5 msec. duration was applied to the ulnar nerve at the axilla, elbow and wrist, and evoked myograms were picked up from the hypothenar eminence muscles.

The spinal reflex time was measured as the latency of the H wave which was recorded from the gastrocnemius muscle by stimulating the posterior tibial nerve at the popliteal fossa.

Room temperature was carefully kept at 30°C during the experiments.

(1) The average maximum conduction velocities of ulnar nerve fibers were 67.0 ± 2.5 meters per second in the forearm and 67.6 ± 2.6 meters per second in the upper arm in the control group and 66.5 ± 2.3 meters per second in the forearm and 67.2 ± 2.6 meters per second in the upper arm in the athletes group.

It was noticeable that any significant differences were recognizable neither between the data of the forearm and upper arm in the two groups nor between those obtained in the athletes group and the control group.

(2) No significant difference as to the spinal reflex time and the spinal reflex index was seen between the two groups, judging from the patterns of their distribution depicted in the graphs. The spinal reflex index is an index which corresponds to the mean conduction velocity of the impulse over the reflex arc.

(3) Distinct individual differences in the maximum conduction velocity of ulnar nerve fibers were observed in both groups. However, no factor was found which might be related to the individual differences from the viewpoint of anthropometric measurements and skillfulness in sport.

From these results, it may be said that no kind of physical exercise increases in the conduction velocity of the nerve fibers in young adults.

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The Histochemical Studies on Leucine Aminopeptidase Activity of Human Gastric Cancers

By

Syun Hosoda and **Sadao Takase**

Department of Pathology, Tohoku University School of Medicine, Sendai; Director : Prof. Dr. K. Akazaki

and

Koichi Yoshida

Department of Surgery, Tohoku University School of Medicine, Sendai; Director : Prof. Dr. M. Muto

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INTRODUCTION

The question of the invasiveness, biological feature characterizing the cancer cells, has been inquired into by many authors from the angles not only of morphology but also of the diffusing factors produced by the cancerous tumor, but still, the solution is apparently hidden behind a thick veil.

In 1956, Burstone, M.S.[1] studied the aminopeptidase activity (AA hereunder) of the cancerous tissues by a technique of his own invention[2], from histochemical standpoint and found that the AA is intensified in the stromatic cells of the infiltrating foci; on this assumption, he announced that the invasiveness of cancer can be interpreted as an effect of this enzyme. Last year, however, Nachlas, B.M. *et al.*[3], upon follow-up study following Burstone's technique and histochemical investigation of the enzyme using 1-leucyl-4-methoxy-2-naphthylamide as substrate, arrived at results utterly contradicting the conclusion obtained by Burstone. According to Nachlas *et al.*, the intensification of AA is not limited to the stroma of the foci of infiltrating cancer cells, but also occurs in activated stroma cells as well, as they demonstrated in the granulation tissue of healing wounds in rats.

The authors have been engaged in studying the process of transformation of the various cells coming forth in the subcutaneous connective tissue under the various stimulations, and have been given the opportunity of obtaining specimens of resected gastric cancer with the least possible delay, by courtesy of the Surgical Clinics of Prof. Muto and Prof. Katsura, Tohoku University Hospital,

細田 峻，高瀬貞夫，吉田弘一

enabling us to examine the enzyme reactions in these specimens. The enzymes detected consisted in 11 varieties, including aminopeptidase to which alone we will confine ourselves in reporting some observations arrived at in our study hereunder.

EXPERIMENTAL MATERIALS AND METHOD

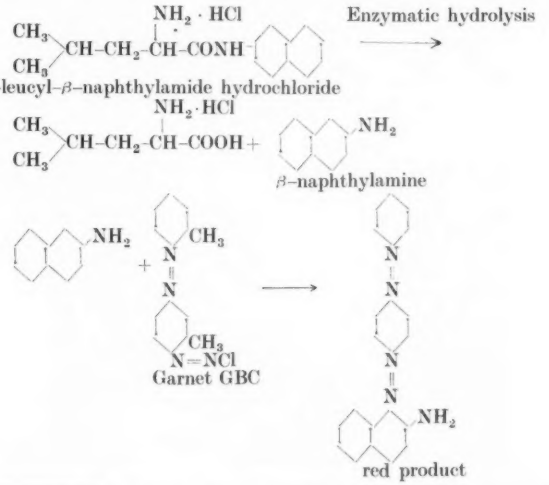
a) Human specimens: Slices of about 1 mm thickness were sampled within ca. 1 hour after extraction from specimens of 30 gastric cancer and 2 gastric ulcer extracted from human subjects by surgery, immediately immersed in 10% buffered neutral formalin, kept in ice-box for fixation for 2 hours, cut into ordinary frozen sections 10–15 μ thick, kept immersed for 30 min. at room temperature (18°C) in a substrate of the composition shown in the following table.

Following incubation with subststrate the sections were washed for several minutes in distilled water, and then mounted on slides. After drying the slides were counter-stained for 15 minutes with Carazzi's hematoxylin.

If counter stained they were washed in running tap water for 10 minutes and mounted in the glycerol.

L-leucyl-β-naphthylamide hydrochloride	10 mg*
distilled water	20 ml
ph 7.2 0.2 M Tris buffer	5 ml
Fast Garnet G B C Salt	15 mg

This mixture was shaken, filtrated into a coplin jar and used at room temperature (18°C). The reaction is illustrated in the following figure.



* We express our sincere thanks to Eizai Chemicals, Chemical Division, for their kind offer of the synthesized preparation.

b) Subcutaneous tissue of the back of rabbits: Normal rabbits were injected with 0.2 cc of typhoid vaccine beneath the dorsal skin and the subcutaneous tissue at the side of injection was sampled from 12 hours to 10 days after the injection to make stretched preparations, which were tested for their AA, as the cancer specimens above, and the results were studied in comparison with the results of the results of similar tests in stretched and dried specimens of normal subcutaneous tissue.

RESULTS

Tab. I shows the AA in the 30 specimens of human gastric cancer and the 2 specimens of human gastric ulcer, together with the histological diagnosis, results of mucinous staining, Borrman's type, invasiveness, Imai's CPL type and metastasis to lymph nodes where detected, of the gastric cancer. The AA in the normal mucous membrane adjoining the foci is listed in Tab. II (cf. also Figs. 1 and 2).

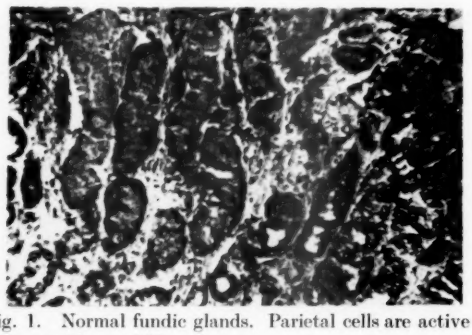


Fig. 1. Normal fundic glands. Parietal cells are active while others are feeble. ×250.

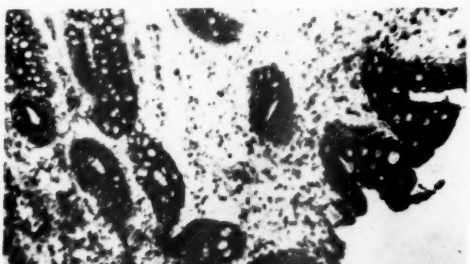


Fig. 2. Normal mucous epithelium of pylorus. The predominant activity is localized on the free border of the lining epithelium in the pyloric area. ×100.

TABLE I. Types of Cancer and Aminopeptipase.

TABLE I. Types of Cancer and Aminopeptipase.

Case No	Borrman type	C.P.L classification	Infiltrating grade	Metastasis* in lymph nodes	Histological diagnosis	Mucinous staining	Aminopeptidase activity		
							Cancer cells		Stroma
							Inner surface of cancer	Infiltrating cancer cells	
1		PIIILO	±++		C. simpl. scirrhosum	+	+	+	±
2	II		+++	L I	〃 〃	+	+	+	±
3	II	PIILO	++	L O	〃 〃	++	++	++	±
4	II	PIILIII	+++	L I	〃 〃	++	++	±	±
5	III	PIILO	++	L O	〃 〃	++	++	++	±
6	II	PIILO	++	L O	〃 〃	++	++	++	±
7	II	PIILII	+++	L I	〃 〃	−	±	−	±
8	III	CLII	+++	L II	〃 . medullare	−	+	−	±
9	I	CLO	+++	L I	〃 . 〃	−	−	−	±
10	II	PIILO	+	L I	〃 . 〃	−	+	−	+
11	IV	PIIILIV	±±+	L II	〃 . 〃	−	+	+	±
12	III	PILIII	+++	L I	〃 . 〃	−	±	±	±
13	III	PILIV	+++	L II	〃 . mucocell.		+	−	±
14	I	PILO	+	L I	〃 . 〃	++	++	+	+
15	II	CLII	+++	L I	〃 . 〃	+++	++	+	±
16	II	CLO	+++	L II	Adenoc. cuboepi. muco	+++	++	+	±
17	III		+	L O	〃 . cuboepithel		±	−	±
18	II	PIILI	+++	L II	〃 . 〃	−	+	+	+
19	III	PILI	+++	L O	〃 . cuboepi. medu	±	+	+	+
20	II	CLV	+++	L I	〃 . cuboepithel	+	+	+	+
21	IV	PIILII	+++	L II	Adenoc. cuboepithel	+	±	+	+
22	II	PILO	+++	L II	〃 〃	+	++	+	+
23	III	PIILII	++	L I	〃 〃	+	+	+	±
24	II	PILIII	+++	L II	〃 . cuboepi. muco	+++	++	+	+
25	II	CLO	+++	L I	〃 . cuboepithel	±	+	+	+
26	III	CLO	+++	L I	〃 . cuboepi. medu	−	−	−	±
27	III	PILO	+++	L I	〃 . cylindroepith	++	++	+	−
28	II	PILIII	+++	L O	〃 . 〃	++	++	+	−
29	II	CLI	+++	L I	〃 〃	++	+	++	±
30	III		+++	L I	〃 . cylind. mucocel	++	+⊥	+	±
31					Ulcus ventriculi				+⊥
32					〃 〃				+⊥

* L O : No metastasis in lymph nodes. L I : Metastasis in primary lymph nodes. (Aa. gastricae, Aa. gastroepiploicae) L II : Metastasis in secondary lymph nodes. (A. hepatica, A. lienalis)

TABLE II. Aminopeptidase Activity of Normal Mucous Epithelium of Stomach

		Aminopeptidase activity	Intracellular localization
Body of the stomach	Mucous epithelium	+	Striated border
	Zymogenic cells	±	Diffuse
	Parietal cells	+	Diffuse
	Mucoid cells	±	Diffuse
Pylorus	Mucous epithelium	+++	Striated border
	Pyloric glands	−	——

The most notable fact observable in looking through Tab. I is that AA is intense in cancer cells showing positive mucicarmine reaction in most cases. This shows that the more active the mucus secretion from the cells in simple as well as adenomatous cancers, the higher rises the AA in the cells. For example, in the specimen No. 9 which belongs to medullary carcinoma in histological classification and the cancer cells in which show obvious anaplasia from normal epithelium of the gastric mucosa, the mucicarmine reaction tests showed utterly negative results, and here the AA was equally negative, while in the specimen No. 4 and No. 6, histologically of scirrhous type, the cells are subject to advanced mucous degeneration, and AA in these cells was found intensely positive (Figs. 3 and 4). In the specimen No. 18 (adenomatous cancer of cubic epithelial cells), the mucus reaction gave negative results and the AA was comparatively

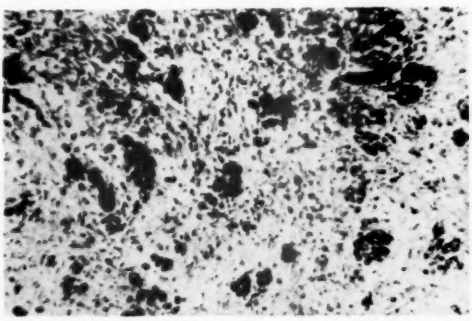


Fig. 3. Scirrhus accompanying strong proliferation of stromal cells. Individual cancer cells stained intensely, while the stromal cells show slight activity. ×250.

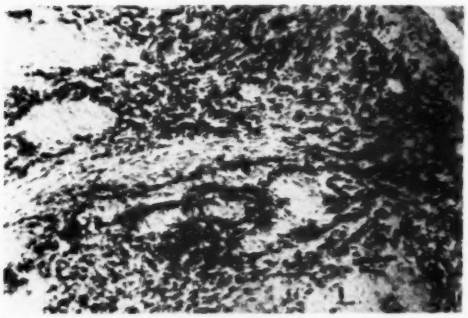


Fig. 4. The same scirrhus as Fig. 3. Aminopeptidase activity of cancer cells enhances, but stromal cells are slightly active. ×100.

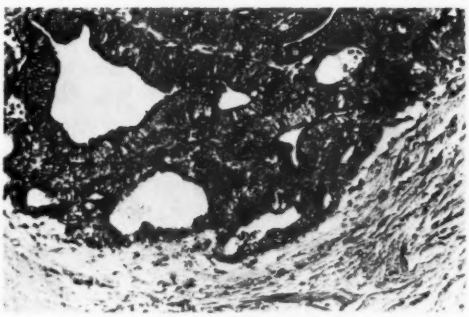


Fig. 5. Columnar cell cancer resembling normal gastric gland. Intense aminopeptidase activity is confined to the luminal borders of the lining cancer cells. ×250.

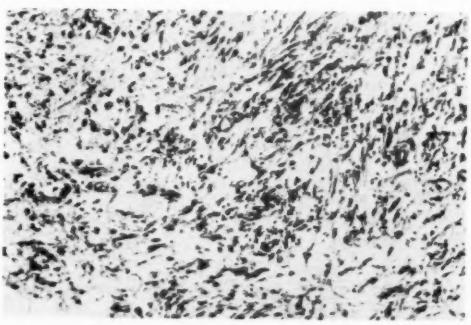


Fig. 6. Numerous proliferating connective tissue cells near the fundus of the gastric ulcer. Mononucleated rounded cell and spindle-like cells show the predominant activity. ×250

feeble. In specimen 29 (a well differentiated cylindrical epithelial cancer), the cancerous structure was very closely similar to that of a normal mucosal epithelium, except that the cells showed invasive growth; a membrane of mucus was found just on the free border of the cell body and AA was strongly positive also exactly in this part (Fig. 5).

The next point to be noted is the low frequency of AA in the stroma, for stromatic AA was found positive in only 3 of the 15 cases of simple cancer (1 of these showed mucus reaction) and 5 of the 15 cases of adenomatous cancer. The frequency in the latter cases was somewhat larger, but the total number of cases is too small to speak on the significance of the difference. The intensity of AA in cancerous stroma gave us the impression of being rather low, as may be well illustrated citing the findings in the control cases of gastric ulcer (specimens 31 and 32) in comparison: In these 2 cases, we found granulation tissue in hyperfunctional stage with intense cellular reaction and new vascular formation in it formed in the ulcerous stroma, and AA was far more prominent in the cells of such a granulation tissue than in normal stroma (Fig. 6).

In comparison of AA in such inflammation foci, the same in the cancer-invaded stroma is feeble, especially so in the stroma of scirrhous cancer. From this finding, it may be deduced that the reaction of the stroma against invasive proliferation of cancer cells has very little of the defensive character, as observable in the stromatic reaction against inflammation. In other words, this means that the irritation exerted by cancer cells on the stroma is much milder than that of inflammation.

The last point to be noted is that AA showed no difinite correlation with any of the Borrman's types or CPL types, the extent of cancerous invasion or the

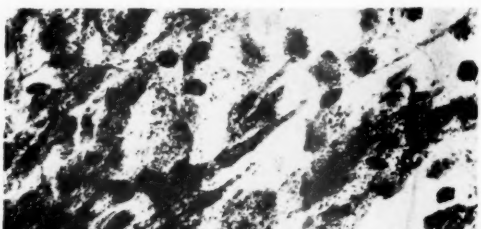


Fig. 7. Subcutaneous connective tissue spread 5 days after subcutaneously 0.2 cc typhoid vaccine injection into the back of the rabbit. Free and rounded histiocytes and elongated stimulationtype fibrocytes have clear positive granules in their cytoplasms. ×250.

existence or nonexistence of metastasis to lymph nodes.

For reference's sake, the results of the above described experiment with rabbits are summarized in the following.

1) In stretched preparations of the subcutaneous tissue of normal rabbits, AA was found clearly positive in the histiocytes and feebly positive in the fibrocytes.

2) Five days after injection of 0.2 cc of typhoid vaccine, numerous free and rounded histiocytes and elongated stimulated-type fibrocytes were found at the site of injection, and the AA in these cells was found intensified over that in normal cells (Fig. 7).

DISCUSSION

Burstone, M.S. reports that, in his tests for AA in various malignant tumors, the stroma showed stronger AA than the cancerous cells in epitheloid as well as hepatic cancer, and concluded on the parallel relation between the invasiveness of the tumor cells and the intensity of the stromatic AA, but in the results obtained by Nachlas, B. M. in his 2 cases of gastric adenocarcinoma, the AA in the stroma was indeed found stronger than in the tumor cells, in agreement with Burstone's idea, but in his cases of mammary cancer, the AA of the tumor cells is marked (++) and that of the stroma (+) ,while in his cases of fibrosarcoma, the AA of tumor cells and the stroma are evaluated as (++) and (−), respectively, denying the parallelism between the invasiveness of the tumor cells and the AA in the stroma, as advocated by Burstone.

In our study, we could observed significant correlation between the AA and the mucus reaction of the tumor cells, but no ovbious correlation between the AA of the tumor cells and of the stroma. Namely, the AA of the tumor cells was found to show individually variable intensity, as far as was observable in our specimens, and this inequality in AA intensity cannot be explained by citing the current criterion of simple cancer versus adenomatous cancer. The AA of the stroma in our cancer specimens was usually found to show merely a feeble positivity, somewhat in disagreement with the above cited authors. This low AA in cancerous stroma was definitively demonstrated by studying the AA in the stroma of ulcer specimens in collation.

As already given in Tab. II, a normal mucosal epithelium of the pylorus has a thin lager with strong AA and mucus reaction positivity just on the free border of the cells, but in some cases (e.g., specimen No. 29), cancer cells very closely approximating normal mucosal epithelial cells were found luxuriantly proliferated in the muscle layer and the submucosa, and the AA and the mucus reaction of these cells also showed close resemblance in distribution to that in the cells of mucosal epithelium of normal structure. In specimen No. 20, the AA and the mucus reaction are of moderate intensity in the cubic epithelial cells forming

the adenocarcinoma, and in specimen No. 9, the cells in the indifferentiated simple medullary cancer showing strong anaplasia were entirely devoid of AA and mucus reaction. Thus, we could find cancer cells of step-wise transitional types in respect of the intensity of AA. Accordingly, we think that this enzyme aminopeptidase is an inherent element in the gastric cancerous cell but find it hard to concur in the opinions that this is produced in the invasion under co-operation of cancerous cells. Since we have found that AA was strongly positive in our 2 specimens of gastric ulcer and the acute inflammation foci caused by injection of typhoid vaccine in rabbits, but weak in the stroma in invasive cancerous tissues, as stated above, we are rather inclined to conclude that this enzyme is unrelated with the invasive property of cancer, as Nachlas *et al.* opined, but is produced by the fibroblastic activity (or, more strictly speaking, we should say the histiocytic-fibrocytic activity) inherent to the stroma, as Nachlas *et al.* would have it.

Since our specimens were fixed in 10% cold buffered neutral formalin for about 2 hrs., we have to consider the possible loss of AA of the stroma during this process, but as Nachlas *et al.* have denied any perceptible influence of brief formalin treatment on the aminopeptidase activity and our control specimens of gastric ulcer similarly fixed did not fail in showing strong AA postivity, we may be justified in neglecting this possibility.

CONCLUSION

1. Upon histochemical examination of the AA in 30 specimens of gastric cancer tissues, it has been confirmed that the aminopeptidase activity and the mucus reaction of the cancer cells rise and fall in intensity in nearly parallel relation. This suggests that the mother cells producing cancer cells are originated in the epithelial cells of the gastric mucous memberane.

2. No definite correlation could be established between the aminopeptidase activity of the gastric cancer cells and the histological diagnosis, Borrman's type or Imai's CPL types of cancer, the invasiveness of the cells or the cancerous metastasis to the lymph nodes.

3. The aminopeptidase activity of the stroma in our 30 cancer specimens was generally feebler than that in the control specimens of gastric ulcer and the local inflammation foci in rabbits injected with typhoid vaccine. This seems to suggest that the irritation to the stroma due to cancer cells is comparatively mild finding of interest when considered in correlation with the frequency of scirrhous cancer in the stomach.

Acknowldegement

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Organization of Tactile Dermatomes in Bullfrog

By

Shinjiro Yamamoto and **Toshio Yamauchi**

From the Department of Surgery, School of Medicine, University of Kanazawa, Japan

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INTRODUCTION

The physiological method of determining the "remaining sensitivity" in the hindlimb of the frog was reported by Eckhard[3], Koschewnikoff[6] and Sherrington[9]. Using the same technique, the attempt to map the whole dermatomes was made by Kubo[7] with the toad.

Kuhn[8] was the first who recorded the evoked potentials from the dorsal roots to study the dermatomes in monkeys and cats. This modern method is concerned only with the activity of the primary sensory neurons. Therefore, this method makes possible the reduction of experimental error in mapping the dermatomes in animals with the method of remaining sensitivity, because it is not bothered by variability of the synaptic action and the reflex movement. This method also make possible the study of the dermatome pertaining to a particular modality of sensation such as tactile sensation. The purpose of the present paper is to report the dermatomes of tactile sensation as determined by the evoked potential technique with the bullfrog.

METHOD

The frog has eleven pairs of the dorsal roots. However, the first pair exists only in the foetal period. Therefore, a series of the dorsal roots from the second to the eleventh pair participate in the spinal sensory innervation. In the present paper, the dorsal roots will be numbered by such genetic nomenclature.

The experiments were carried out on the bullfrog (*rana catesbiana* Shaw). The animal was anesthetized by hypodermal injection of urethane of 1–1.6 g/kg body weight. Laminectomy was carried out routinely and the spinal cord was exposed. The action potentials ascending through the dorsal roots of the third to the eleventh pair were recorded by a wire electrode hooking up the dorsal root to be examined. Care was taken to isolate the hooked-up root from surrounding tissues as much as possible by drying the root. The indifferent electrode was put on nearby tissues. Recording of neural activities from the cranial nerves

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(trigeminus and vagus) or from the second dorsal root was carried out by the use of a fine needle electrode, because it was difficult to apply a hook-type electrode to these nerves. The electrode was made of a silver needle insulated with glass as designed by Yamamoto *et al*[12]. The tip diameter of the electrode measured in the range of 10–20 μ. Since the needle electrode used in the present experiment was so fine that picked-up discharges were almost unitary in nature, and the skin area activating the discharges was restricted, a single insertion of the electrode was not enough to map out the whole dermatome of the nerve under examination. Therefore, the dermatomes belonging to the second spinal root and the cranial nerves were constructed by repeated insertions of the needle electrode.

With the use of amplification equipment, action potentials were recorded by an electromagnetic oscillograph. They were also seen on an oscilloscope and heard by a loud-speaker. When the background noise was big relative to the evoked potentials, the employment of a loud-speaker was most useful for discriminating the receptive field of the root. Afferent impulses were initiated by brushing the skin with a small paint brush or by a slight touch with the tip of a glass rod. All the stimuli were given thus manually. The receptive field was divided into two concentric areas from center to fringe such as a contour map in the order of the strength of afferent discharges evoked. The cutaneous area innervated by each root or nerve was sketched precisely or photographed, after marking it with red ink on the skin of the animal.

RESULTS

The nerves which innervate the skin of the bullfrog were the trigeminal nerve, the vagus nerve and the second to the eleventh spinal dorsal roots. The eleventh dorsal root was the most suitable object for this sort of investigation, because of its relatively small number of fibers included and a long course in the vertebral canal. An example of records taken from the eleventh root is seen in Fig. 1. Two kinds of spikes, big and small ones, were found. The big spikes were about three times as high in amplitude as the samll ones. The former was induced only by tactile stimulation of the skin and found to adapt rapidly. The small spikes were seen even in a resting state and found to increase in frequency, adapting slowly, mostly upon mechanical stimulation of the cloaca such as distension of it. They also somewhat increased the discharging frequency in response to a touch stimulus on the skin around the anal area. The skin area within a semicircle around the anus and the proximal half of postaxial side of the thigh were innervated by this root. The density of the innervation decreased as the distance from the anus increased, and the fringe zone was intensely overlapped by the tenth and the seventh dorsal roots. It should be noted that the skin area of the sphincter of the anus was innervated by the roots of both sides. This was the only portion where the crossed overlap was found in the areas innervated

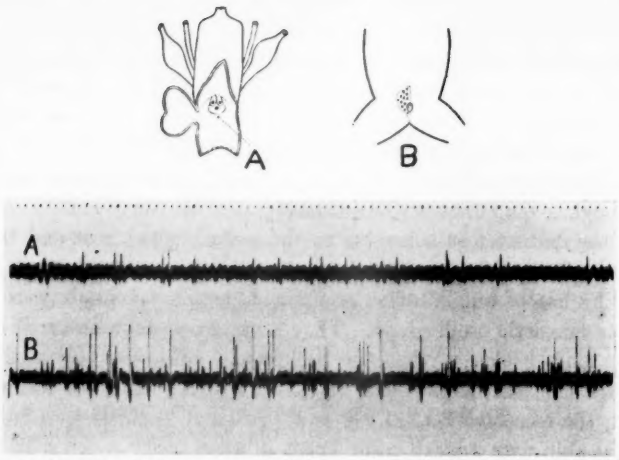

Fig. 1. Impulse discharges recorded from eleventh dorsal root of bullfrog.

A: discharges induced by distension of cloaca. Cloaca was exposed by incision of skin and removal of urostyle. Stimulated portion was indicated by "A" of upper diagrams.

B: discharges induced by tactile stimuli. Brushing was applied to portion indicated by "B" of upper diagrams.

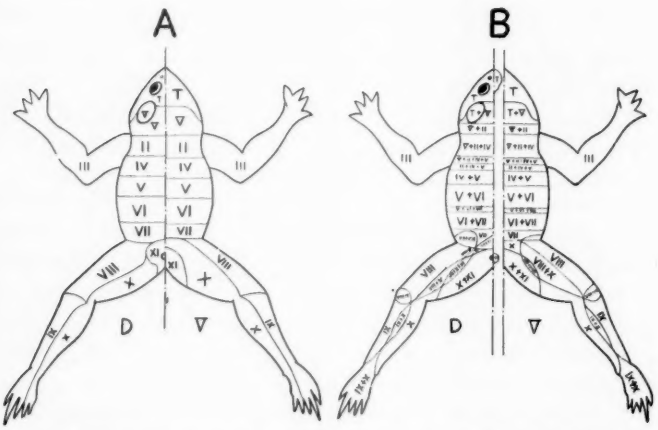

Fig. 2. Dermatome chart of bullfrog mapped by evoked potential technique.

A: appropriate delimitation of dermatomes. This was made by combining central area of each dermatome.

B: mode of overlap of each dermatome.

D and V: dorsal and ventral aspects of bullfrog; T: trigeminal nerve; V: vagus nerve; II to XI: second to eleventh spinal dorsal roots.

by the spinal nerves.

The central areas of the dermatome which gave rise to most active discharges were thus determined for each root and nerve, and mapped out. This is shown in Fig. 2A. The overlap of the dermatomes of neighbouring roots was considerable. The mode of overlap is diagrammed in Fig. 2B. Though there were some individual variations in the extent of the dermatome with respect to the same segmental level, they were generally found to be slight, so that figures were drawn from the averaged result of a series of thirty experiments.

As seen in Fig. 2, the skin of the hindlimb is innervated by the last four dorsal roots. The tenth dorsal root was found to innervate the area in postaxial side of the thigh and the leg, and the whole foot. As described previously, this overlapped innervation of the eleventh dorsal root in the region of the anus. The ninth dorsal root innervated the whole foot and the preaxial side of leg and knee. The eighth dorsal root innervated the preaxial side of thigh and knee. Overlapped innervation was most marked in the areas around the anus and in the most distal part of the hindlimb.

The trunk was found to be innervated by a series of the dorsal roots from the second to the seventh excepting the third one in the order of rostral-caudal direction. The overlap of the root innervation was found more marked than in the case of the hindlimb. A considerable area of the rostral part of the trunk was also overlapped by innervation of the vagus nerve. The vagal innervation extended to skin areas of the second and the fourth roots, and partly to that of the fifth root. It is to be noted that the forelimb was found innervated exclusively by the third dorsal root which did not participate in innervation of the trunk.

The rostral part of the head including the mouth was found innervated by the trigeminal nerve. The fringe area of trigeminal innervation extended to the caudal part of the head, and this was overlapped by innervation of the vagus nerve. It should be noted that the portion around the nostril was innervated by the trigeminal nerves of both sides.

The cutaneous innervation of the vagus nerve was found strikingly extensive. The central area innervated by the vagus nerve was of a band form at the level of the ear membrane. As described previously, its fringe zone was wide and covered the whole shoulder girdle area overlapping the territories of the second, the fourth and partly the fifth spinal roots.

DISCUSSION

Sherrington's studies on dermatomes[9,10] were based upon the method of "remaining sensitivity". These studies for the first time adequately demonstrated a shifting overlap between consecutive adjacent dermatomes. The overlap was found to be most extensive at the apices of the limbs, whereas any given

area of the skin on the trunk was usually noted to be innervated by not more than three dorsal roots. These findings have been corroborated by other workers in animals and in man (Cardozo[1], De Boer[2], Foerster[4], Kubo[7], and Van Rijnberk and ten Cate[11]). In contrast to these findings, the overlap of the dermatome in the bullfrog was found to be most extensive in the trunk, especially in the shoulder girdle area. The trunk was innervated by two or more nerve roots. In the shoulder girdle area, the cutaneous innervation of the vagus nerve intermingled widely with the spinal innervation. The apex of the hindlimb was overlapped by innervation of both the ninth and tenth roots. However, the areas in the thigh and leg innervated by the eighth, the ninth and the tenth roots were found to have no marked overlap. This was already demonstrated by Koschewnikoff in 1868.[6] As far as the forelimb is concerned, it was innervated exclusively by the third dorsal root. No other root invaded the forelimb and the third root did not interfere with the innervation of the trunk.

The higher the species of vertebrates in the phylogenetic scale, the more marked the shifting of dermatome is. The most primitive form of the dermatome is considered to be of a band form. This is seen in a lower vertebrate such as the pleuronectid (De Boer[2]). In the trunk of the frog, the shifting of the dermatome is limited and the band form is still preserved by each dermatome. However, the shape of dermatome is markedly complicated in the hindlimb.

The present study shows that each sensory spinal root and cranial nerve possess a cutaneous field consisting of a central area producing strong centripetal discharges and a peripheral fringe yielding weaker responses. The fringe zone varies in width and is intensely overlapped by the adjacent roots. The fringe zone is particularly narrow where it borders the middorsal and midventral lines of the trunk and the axial lines of the hind limb. This is also true with the monkey and the cat (Kuhn[8]).

Sherrington[9] found that the crosslap of right- and left-hand nerves was especially marked in the penis in the monkey. In the present study, the crossed-over innervation was found around the nostrils and the anus of the bullfrog. It is of great interest that such a crossed overlap of the sensory nerve occurs at both ends of the metamery, i.e. the trigeminal nerve and the eleventh spinal dorsal root.

Recent studies on the dermatomes of man (Keegan[5]) demonstrate that the areas of the extremities innervated by each dorsal root is continuous to the middorsal line or to the midventral line of the trunk, without being isolated as an island as has been demonstrated by previous workers. However, this was not established with the hindlimb in the cat and the monkey (Kuhn[8]). In the present investigation, it was found that the areas innervated by the third, the eighth and the ninth dorsal roots are not continuous to either the middorsal line or midventral line of the trunk. Therefore, it is seen in the dermatomal construction of the trunk that the second and the fourth roots neighbour each other

without being spearated by the third root. A similar situation can be found between the seventh and the eleventh roots at the caudal end of the trunk.

The cutaneous innervation of the vagus was found strikingly wide. It distributes from the level of the ears to the shoulder girdle area. It is well known that vagal contribution to skin sensation in man is limited in the vicinity of the tympanic membrane. Therefore, it may be suggested that the component of somatic sensation of the vagus nerve becomes smaller in the phylogenetic development of vertebrates.

SUMMARY

The evoked potential technique was used to investigate functional organization of the spinal dorsal roots and the cranial nerves in the bullfrog. Afferent stimuli were initiated by brushing or touching on the skin area, and cutaneous tactile fields were mapped. The overlap of innervation was found more marked in the trunk rather than in the extremities. A crossed-over innervation was found in the skin areas surrounding the nostrils and the anus. The area innervated by the vagus nerve extended from the occiput to the shoulder girdle area, and overlapped extensively the area innervated by the trigeminal nerve and the spinal dorsal roots.

Acknowledgment

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